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13. ABSTRACT (Maximum 200 Words)

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues. However, not all cancers are sensitive to TRAIL-mediated apoptosis. Thus, TRAIL-resistant cancer cells must be sensitized first to become responsive to TRAIL. In this study, we observed that pretreatment of acetyl salicylic acid (ASA) augmented TRAIL-induced apoptotic death in human prostate adenocarcinoma LNCaP and human colorectal carcinoma CX-1 cells. Western blot analysis showed that pretreatment of ASA followed by TRAIL treatment activated caspases (8, 9, and 3) and cleaved PARP (poly (ADP-ribose) polymerase), the hallmark feature of apoptosis. Interestingly, at least 12 h of pretreatment with ASA was prerequisite for promoting TRAIL-induced apoptosis and was related to downregulation of Bcl-2. Biochemical analysis revealed that ASA inhibited NF-κB activity, which is known to regulate Bcl-2 gene expression, by dephosphorylating IκB-α and inhibiting IKKβ activity, but not by affecting the HER-2-PI3K-Akt signal pathway. Overexpression of Bcl-2 suppressed the promotive effect of ASA on TRAIL-induced apoptosis and changes in mitochondrial memembrane potential. Taken together, our studies suggest that ASA-promoted TRAIL cytotoxicity is mediated through downregulating Bcl-2 and decreasing mitochondrial membrane potential.

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Introduction :

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutic agents, such as drugs, irradiation, and immunotherapy, since most of their primary cytotoxic effects are through apoptosis (1, 2). After the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

Recent studies have also revealed that TRAIL, which is constitutively expressed on murine natural killer cells in the liver, plays an important role in surveillance of tumor metastasis (3). The apoptotic signal of TRAIL is transduced by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF- receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling (4-7). Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif (8). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, anti-tumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (9, 10).

TRAIL has been considered a new therapeutic agent, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (10-13). However, many tumor cells have been shown to be resistant to TRAIL (14, 15). Several researchers have reported that various sensitizing agents like chemotherapeutic drugs (16, 17), cytokines (18), and matrix metalloprotease inhibitors (19) are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

In recent studies, nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetyl salicylic acid (aspirin; ASA), have been used as chemopreventive agents of cancers to induce apoptosis or reduce the incidence of tumor formations in a variety of organs, i.e., colon (20), lung (21), stomach (22), and colorectum (23). ASA is known to act by directly suppressing cyclooxygenase enzyme (COX-1 and COX-2), the rate-limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. ASA was also shown to be effective in inhibition of ultraviolet radiation and carcinogen-induced tumor formations in animal models (24, 25).

In this study, we examined whether ASA in combination with TRAIL increases TRAIL-induced apoptotic death in TRAIL-resistant human cancer cells. We hypothesized that pretreatment with ASA enhances TRAIL-induced apoptosis by promoting the mitochondrial-dependent apoptotic pathway. Our studies demonstrate that ASA augments TRAIL-induced apoptosis by downregulating Bcl-2 gene expression and decreasing mitochondrial membrane potential, which subsequently leads to an increase in caspase activation.

Body :

The long-term goal of our research project is to develop a novel therapy for HER-2/neu overexpressing prostate cancer. Previous studies have shown that the HER-2/neu homodimer constitutively activates the PI(3)K-Akt-NF- B signal transduction pathway. In this budget period, we examined whether the PI3K-Akt-NF- B signal transduction pathways is involved in TRAIL-induced apoptosis. As a first step we investigated whether modulation of the PI3K-Akt -NF- B signals affects TRAIL-induced cytotoxicity. As DU-145 cells were treated with acetyl salicylic acid (ASA: aspirin), an inhibitor of IKK , we observed that TRAIL-induced cytotoxicity was promoted. Our observations are illustrated below:

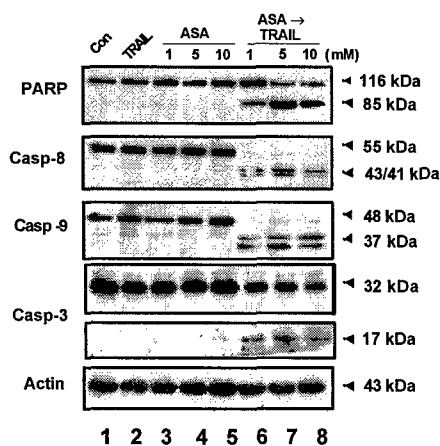### *Acetyl salicylic acid (ASA) promotes TRAIL-induced cytotoxicity*

To investigate the effect of ASA on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma LNCaP cells were pretreated with ASA and treated with TRAIL in the presence of ASA. Figures 1A and 1B show that little or no cytotoxicity was observed with 1 mM ASA alone or 200 ng/ml TRAIL alone. However, pretreatment of ASA promoted TRAIL-induced cytotoxicity which was dependent upon concentrations of ASA (Fig. 1A) and TRAIL (Fig. 1B). Similar results were observed with TdT-mediated dUTP Nick end labeling (TUNEL) staining (Fig. 1C). Data from TUNEL assays show that apoptotic cell death occurred when LNCaP cells were pretreated with ASA followed by TRAIL.
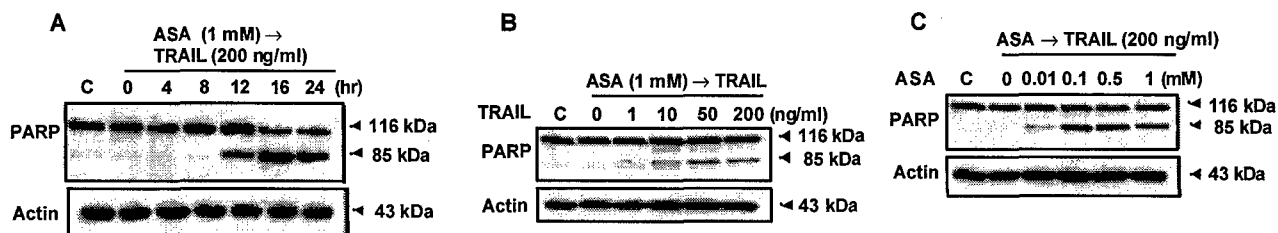
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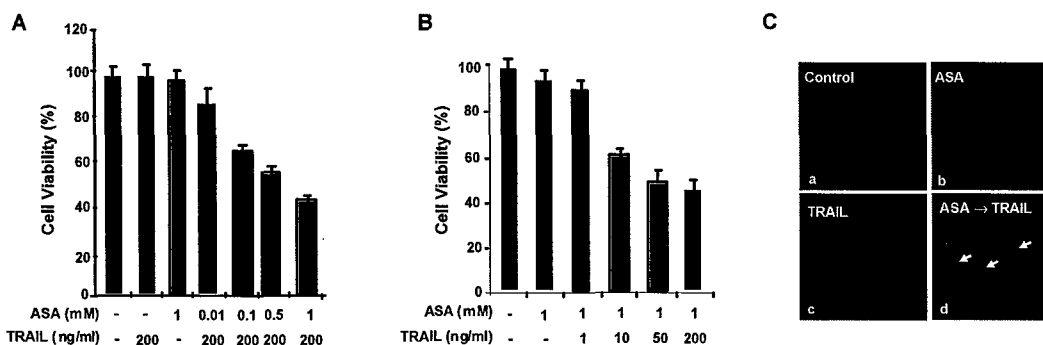
Figure 1. Effect of pretreatment of acetyl salicylic acid on TRAIL-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells. (A) Cells were pretreated with various concentrations of acetyl salicylic acid (ASA; 0.01-1 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. (B) Cells were pretreated with 1mM ASA for 20 h and treated with/without various concentrations of TRAIL (1-200 ng/ml) for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. (C) Cells were pretreated with 1 mM ASA for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by arrows. (a) untreated control; (b) ASA only; (c) TRAIL only ; (d) ASA   TRAIL.

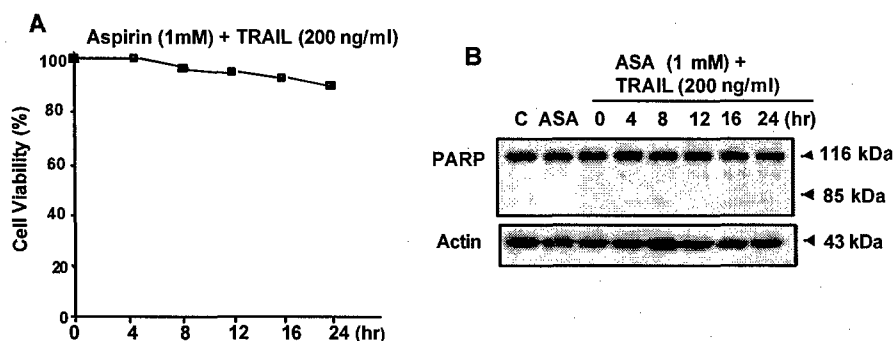## Effect of ASA on TRAIL-induced apoptosis

Additional studies were designed to examine whether pretreatment of ASA followed by treatment with TRAIL causes poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis. Figure 2 shows that PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of 200 ng/ml TRAIL and ASA (1-10 mM). The cleavage of PARP was not observed by treatment with ASA alone or TRAIL alone. These results were similar to the observations of cytotoxicity (Figs. 1A and 1B). Western blot analysis shows that procaspase-8 (55 kDa) was cleaved to the intermediates (41 and 43 kDa) by pretreatemnt with ASA and treatment with TRAIL. The combined treatment of TRAIL and ASA also resulted in an increase in caspase-9 activation as well as caspase-3 activation (Figure 2). The precursor form of caspase-9 and −3 was cleaved to active form 37 kDa and 17 kDa, respectively. ASA alone or TRAIL alone did not activate caspases. We extended our studies to investigate a time course and dose response on PARP cleavage. Figure 3A shows that at least 12 h of pretreatment with ASA was required for PARP cleavage in the presence of TRAIL. Figures 3B and 3C show that miminal 10 ng/ml TRAIL or 0.01 mM ASA was required for PARP cleavage in the presence of 1mM ASA or 200 ng/ml TRAIL, respectively. We further investigated whether treatment with ASA is prerequisite. Figure 4 shows that combined treatment with TRAIL and ASA without pretreatment with ASA caused little or no cytotoxicity and PARP cleavage. Taken together, these results suggest that pretreatment with ASA for 12 h is essential for inducing apoptotic death in the presence of TRAIL.

Figure 2. Effect of pretreatment of acetyl salicylic acid on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP cells. Cells were pretreated with various concentrations of ASA (1-10 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa) and cleaved intermediates (41, 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa) and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

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Figure 3. Effect of pretreatment of acetyl salicylic acid on TRAIL-induced PARP cleavage in LNCaP cells. (A) Cells were pretreated with 1 mM ASA for various times (0-24 h) and treated with 200 ng/ml TRAIL for 4 h. **(B)** Cells were pretreated with 1 mM ASA for 20 h and treated with various concentrations of TRAIL (1-200 ng/ml) for 4 h. **(C)** Cell were treated with various concentrations of ASA (0.01- 1 mM) for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Actin is shown as an internal standard.

Figure 4. TRAIL in combination with acetyl salicylic acid without pretreatment of acetyl salicyclic acid has no effect on TRAIL-induced apoptosis in LNCaP cells. (A) Cells were treated with 200 ng/ml TRAIL in combination with 1 mM ASA for various times (0-24 h). Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **(B)** Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Actin is shown as an internal standard.

Role of COX in TRAIL-induced apoptosis

It is well known that ASA inhibits only COX-1 at low concentrations ($IC_{50} = 44$ M), but both COX-1 and COX-2 at higher concentrations ($IC_{50} = 1100$ M) (29). To examine whether the promotive effect of ASA on TRAIL-induced apoptosis is mediated through inhibiting COX, LNCaP cells were pretreated with various NSAIDs and then treated with TRAIL. Unlike ASA, Figures 5A-5C show that no significant cleavage of PARP was observed by treatment with various concentrations of sulindac sulfide ($IC_{50} = 1.02$ M for COX-1 and $IC_{50} = 10.43$ M for COX-2), NS-398 (a selective COX-2 inhibitor; $IC_{50} = 4.81$ M for COX-1 and $IC_{50} = 0.47$ M for COX-2), or indomethacin (a nonselective COX inhibitor; $IC_{50} = 0.16$ M for COX-1 and $IC_{50} = 0.46$ M for COX-2). To confirm our observations, cells were transfected with COX-2 siRNA or mock siRNA. Figure 5D shows that the expression of COX-2 was effectively inhibited by siCOX-2. However, knock-down of COX-2 expression did not promote TRAIL-induced apoptosis. Nonetheless, pretreatment with ASA promoted TRAIL-induced apoptosis regardless of the presence or the absence of COX-2. These results suggest that COX is not involved in ASA-promoted TRAIL cytotoxicity.
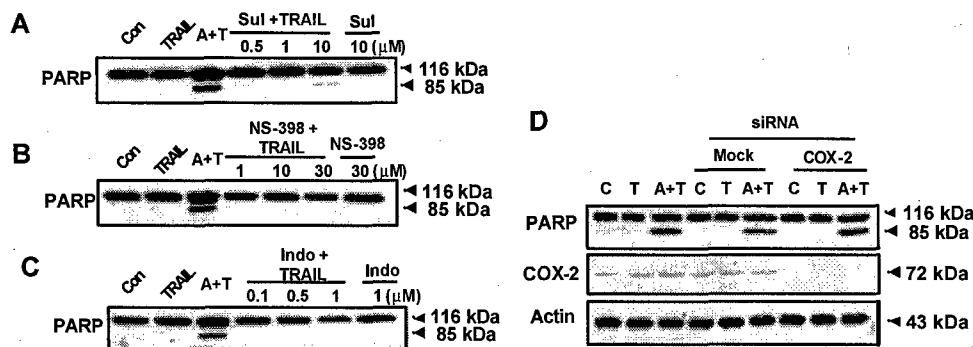
Figure 5. Role of COX in acetyl salicylic acid-induced TRAIL cytotoxicity. (A-C) LNCaP cells were preteated with 1 mM ASA (A + T), or various concentrations of sulindac sulfide (Sul + TRAIL), NS-398 (NS-398 + TRAIL), or indomethacin (Indo + TRAIL) for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. (D) LNCaP cells were transfected with COX-2 siRNA or mock siRNA and incubated for 36 h. Cells were pretreated with 1 mM ASA for 20 h and treated with 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-PARP, anti-COX-2, or anti-actin antibody. C, untreated control cells. T, TRAIL treated cells. A+T, ASA and TRAIL treated cells.

Effect of ASA on the level of TRAIL receptor family and anti-apoptotic proteins

It is well known that TRAIL can interact with death receptors (DR4 and DR5), which trigger apoptotic signals (4). Such signals may be blocked by expression of the antagonistic decoy receptors (DcR1 and DcR2). Previous studies demonstrate that increased DR5 levels are induced by chemotherapeutic agents (30). Thus, we examined whether pretreatment of ASA affects the level of TRAIL receptors and anti-apoptotic proteins, and consequently promotes apoptosis by treatment with TRAIL. LNCaP cells were pretreated with ASA (1-10 mM) and treated with 200 ng/ml TRAIL in the presence of ASA. Data from western blot analysis reveal that ASA treatment did not signficantly alter the levels of the TRAIL receptors (DR4, DR5, and DcR2), and the anti-apoptotic proteins (FLIP$_L$, FLIP$_S$, IAP-1, IAP-2, and Bcl-X$_L$), although it reduced the level of Bcl-2 (Figs. 6A and 6B). The reduction of Bcl-2 during treatment with 1 mM ASA was dependent upon exposure time (Fig. 6B). To confirm the effect of ASA on Bcl-2 gene expression, LNCaP or DU-145 prostatic cancer cells were treated with various concentrations of ASA and expression of Bcl-2 was examined. Figure 7A shows that ASA reduced the level of Bcl-2 in both cell lines. Data from RT-PCR assay in Figure 7B shows that the level of Bcl-2 mRNA was significantly decreased during treatment with ASA. The reduction of Bcl-2 mRNA was dependent upon ASA concentrations. These results suggest that the reduction of Bcl-2 level during treatment with ASA was due to suppression of Bcl-2 gene transcription.
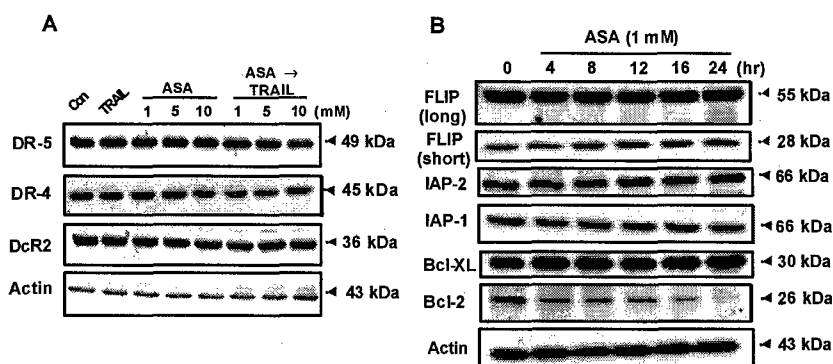
Figure 6. Effect of pretreatment of acetyl salicylic acid in combination with TRAIL on intracellular levels of TRAIL receptors (A) or anti-apoptotic proteins (B) in LNCaP cells. (A) Cells were pretreated with various concentrations of ASA (1-10 mM) for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-DR-5, anti-DR-4, anti-DcR2, or anti-actin antibody. Actin was shown as an internal standard. (B) Cells were treated for various times (4-24 h) with 1 mM ASA and harvested. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-FLIP, anti-IAP-1, anti-IAP-2, anti-Bcl-XL, anti-Bcl-2, or anti-actin antibody. Actin is shown as an internal standard.
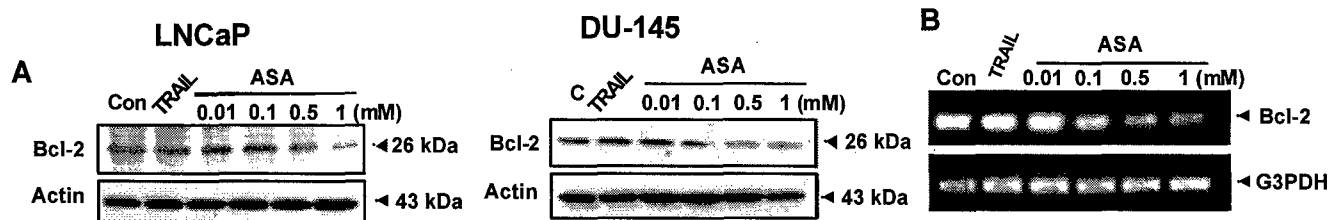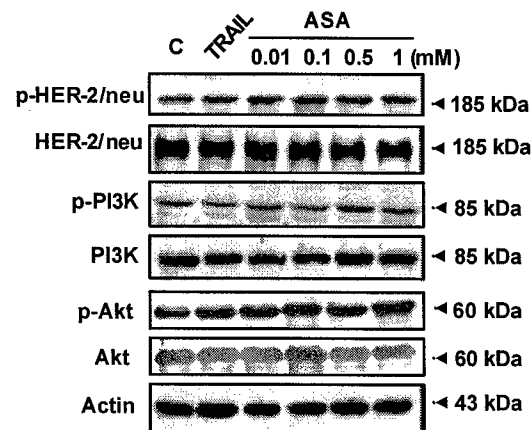

Figure 7. Acetyl salicylic acid-mediated downregulation of Bcl-2 expression in LNCaP or DU-145 cells. (A) Cells were treated with various concentrations of ASA (0.01-1 mM) or 200 ng/ml TRAIL for 20 h for LNCaP cells or 10 ng/ml
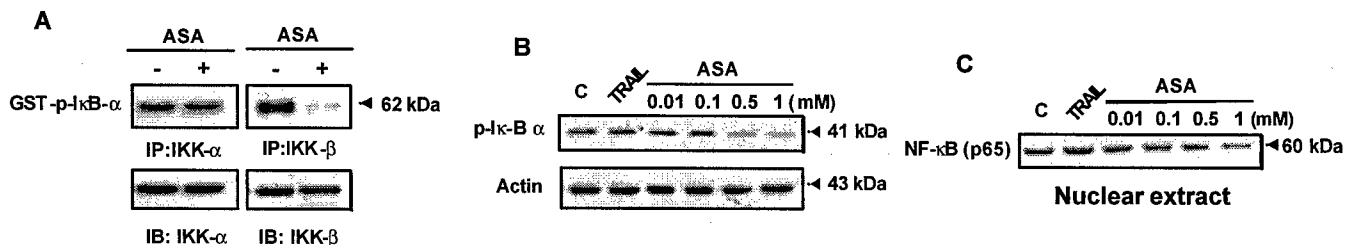
TRAIL for 20 h for DU-145 cells. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-Bcl-2 or anti-actin antibody. Actin was shown as an internal standard. (B) RT-PCR analysis was performed for detecting Bcl-2 or glyceraldehydes-3-phosphate dehydrogenase (G3PDH) expression in LNCaP cells. G3PDH is shown as an internal standard.

Effect of ASA on the HER-2/neu-PI3K-Akt-NF-κB signal transduction apthway

It is well known that Bcl-2 expression is regulated by NF- B, a dimeric transcription factor (31). We postulated that ASA inhibits NF- B activity, which subsequently decreases transcription of Bcl-2. To examine this possibility, the effect of ASA on an upstream signal transduction of NF- B was investigated. Figure 8 shows that ASA treatment did not either change the level of HER-2/neu, PI3K, and Akt, or alter the phosphorylation of these proteins. In contrast, ASA inhibited IKK activity, dephosphorylated I B- , and prevented NF- B nuclear translocation (Fig. 9A-9C). These results suggest that ASA downregulates Bcl-2 gene expression by inhibiting the IKK - I B- -NF- B signal transduction pathway.

Figure 8. Effect of acetyl salicylic acid on HER-2/neu-PI3K-Akt signal transduction pathway in LNCaP cells. Cells were treated with various concentrations of ASA (0.01-1 mM) or 200 ng/ml TRAIL for 20 h. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-HER-2/neu, anti-phospho-HER-2/neu, anti-PI3K, anti-phospho-PI3K, anti-Akt, anti-phospho-Akt, or anti-actin antibody.
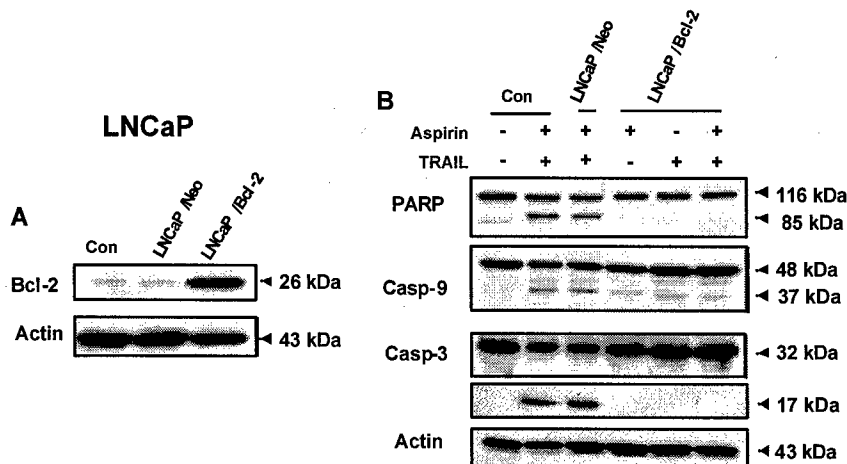

Figure 9. Effect of acetyl salicylic acid on IKK activity (A), IκB-α phosphorylation (B), or NF-κB translocation (C) in LNCaP cells. (A) Cells were lysed and IKK proteins were purified by immunoprecipitation. The purified IKK proteins were incubated with or without 1 mM ASA for 30 min at 4 C and *in vitro* kinase assay was performed at 30 C for 30 min with GST-I B as substrate. (B) Cells were treated with various concentrations of ASA (0.01-1 mM) or 200 ng/ml TRAIL for 20 h and lysed. Equal amounts of protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-phospho-I B- or anti-actin antibody. (C) Cells were treated with various concentrations of ASA (0.01-1 mM) or 200 ng/ml TRAIL for 20 h and nuclear proteins were extracted. Equal amounts of nuclear protein (20 g) were separated by SDS-PAGE and immunoblotted with anti-NF- B antibody.

### Role of Bcl-2 in ASA-enhanced TRAIL cytotoxicity

To determine whether ASA-mediated downregulation of Bcl-2 plays an important role in the augmentation of TRAIL-induced apoptotic death, LNCaP cells or human colorectal carcinoma CX-1 cells were stably transfected with either an empty control vector (pcDNA 3-neo) or vector containing Bcl-2 (pcDNA3-Bcl-2). Figures 10 and 11 show that

pretreatment with ASA followed by treatment with TRAIL caused PARP cleavage, activation of caspases as well as cytotoxicity in control vector transfected cells. However, overexpression of Bcl-2 protected LNCaP and CX-1 cells from ASA-enhanced TRAIL cytotoxicity. These results suggest that ASA-promoted TRAIL cytotoxicity is mediated by downregulating Bcl-2.

Figure 10. Overexpression of Bcl-2 effectively inhibits potentiation of TRAIL-induced apoptosis by acetyl salicylic acid in LNCaP cells. (A) Immunoblot of Bcl-2 expression in control vector-transfected (LNCaP/neo) and Bcl-2 transfected (LNCaP/Bcl-2) single cell clones of LNCaP cells. Con, untransfected parental control cells. (B) Parental control, LNCaP/neo, or LNCaP/Bcl-2 cells were treated with 200 ng/ml TRAIL for 4 h with/without pretreatment of 1 mM ASA for 20 h and then harvested. Cell lysates were subjected to immunoblotting for caspase-9, caspase-3, PARP, or actin. Actin is shown as an internal standard.
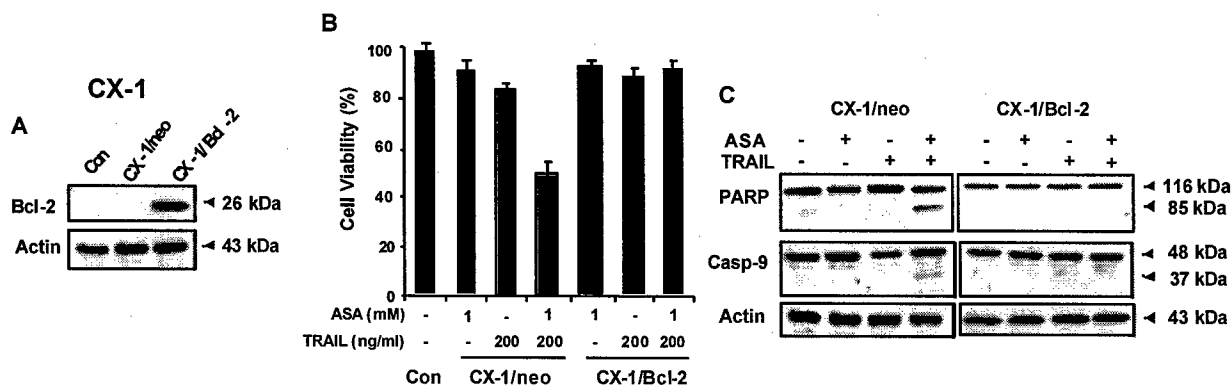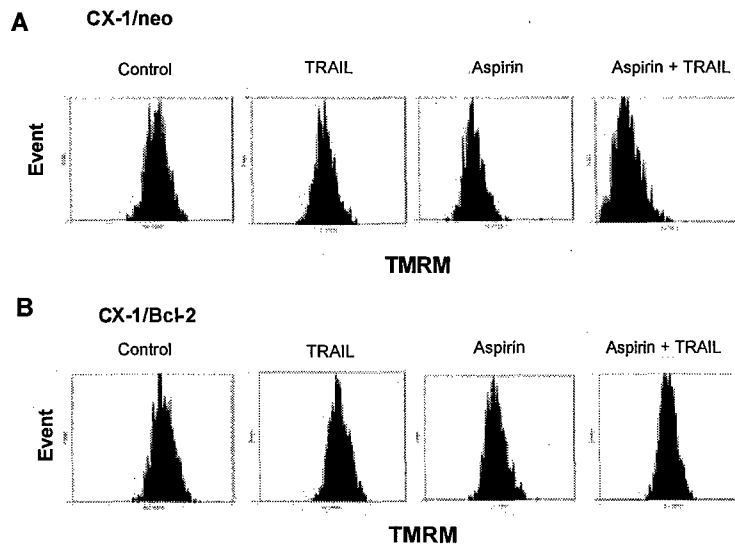

Figure 11. Overexpression of Bcl-2 effectively inhibits augmentation of TRAIL-induced apopotic death by acetyl salicylic acid in colorectal carcinoma CX-1 cells. (A) Immunoblot of Bcl-2 expression in control vector-transfected (CX-1/neo) and Bcl-2 transfected (CX-1/Bcl-2) single cell clones of CX-1 cells. Con, untransfected parental control cells. (B) Cells were pretreated with 1 mM ASA for 20 h and treated with 200 ng/ml TRAIL for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. (C) Parental control, CX-1/Neo, or CX-1/Bcl-2 cells were treated with 200 ng/ml TRAIL for 4 h with/without pretreatment of 1 mM ASA for 20 h and then harvested. Cell lysates were subjected to immunoblotting for caspase-9, PARP, or actin. Actin is shown as an internal standard.

Overexpression of Bcl-2 prevents alteration of mitochondrial membrane potential by treatment with ASA and TRAIL

Bcl-2 is an anti-apoptotic protein that inhibits the release of cytochrome c from mitochondria into the cytoplasm, thereby downregulation of Bcl-2 may promote intrinsic mitochondrial-mediated apoptosis (32, 33). To investigate whether ASA disrupts mitochondrial membrane potential and overexpression of Bcl-2 protects cells from this disruption, CX-1/Bcl-2 or CX-1/neo cells were pretreated with ASA and treated with TRAIL. We used mitochondrial-specific dye tetra-methylrhodamine methylester (TMRM) to measure the mitochondrial membrane potential. Figure 12 shows that

overexpression of Bcl-2 inhibited the loss of mitochondrial membrane potential during treatment with ASA alone or ASA in combination with TRAIL.

A   CX-1/neo

B   CX-1/Bcl-2

Figure 12. Effect of Bcl-2 overexpression on acetyl salicylic acid and TRAIL-induced mitochondrial membrane perturbations CX-1/neo (**A**) or CX-1/Bcl-2 (**B**) cells were pretreated with 1 mM ASA for 20 h and treated with/without 200 ng/ml TRAIL for 4 h. Mitochondrial membrane potential (MMP) was assessed by flow cytometry using the fluorescent dye TMRM.

A model for the effect of ASA on the TRAIL-induced apoptotic pathway

Figure 13 shows a schematic diagram of a model which is based on the literature and our data. ASA blocks the Akt-NF-κB survival signal pathway by inhibiting IKK. The inhibition of this pathway results in suppression of the expression of Bcl-2, an anti-apoptotic molecule.
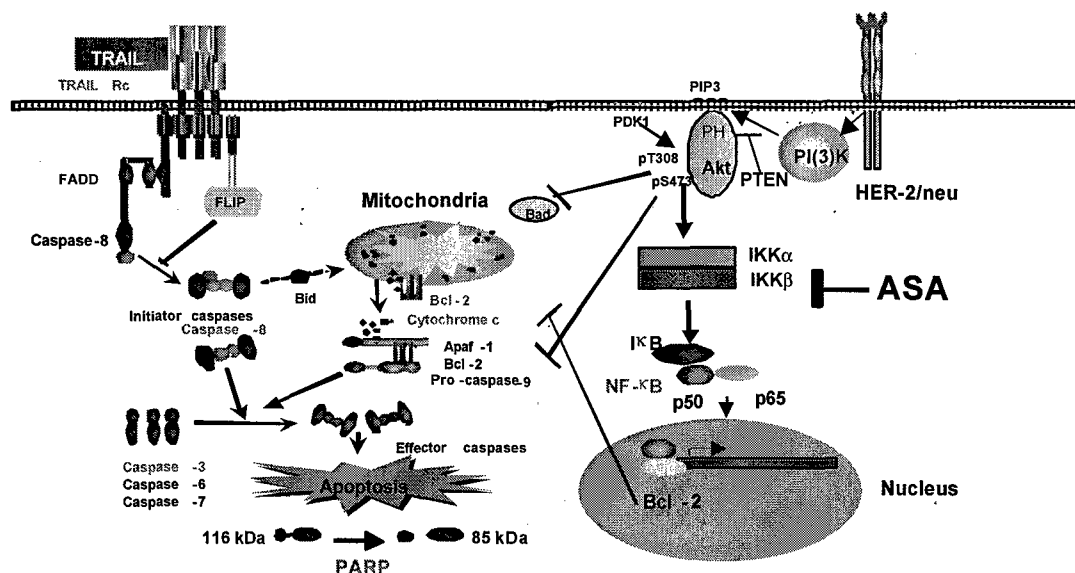
Figure 13. A schematic model for the effect of acetyl salicylic acid on the TRAIL-induced apoptotic death signal pathway.

Key research accomplishments:

We previously proposed that the PI3K-Akt-NF- B signal plays an important role in TRAIL sensitivity. In this study we observed that aspirin enhances TRAIL-induced cytotoxicity in the human prostate carcinoma LNCaP cell line. The mechanism of this enhancement is shown to be almost certainly due to inhibition of IKK kinase by treatment with aspirin. Thus, our data support our proposed hypothesis.

Reportable Outcomes

(1) Kim, Ki M., and Lee, Y.J. : Amiloride Augments TRAIL-induced Apoptotic Death by Inhibiting Phosphorylation of Kinases and Phosphatases Associated with The PI3K-Akt Pathway. Oncogene, 24, 355-366, 2005.

(2) Lee, Y.J., Moon, M.S., Kwon, S.J., and Rhee, J.G.: Hypoxia and Low Glucose Differentially Augment TRAIL-induced Apoptotic Death. Mol. Cell. Biochemistry, 270, 89-97, 2005.

(3) Kim, K.M., and Lee, Y.J. :Role of HER-2/neu Signaling in Sensitivity to Tumor Necrosis Factor-related Apoptosis-inducing Ligand: Amiloride Enhances TRAIL Cytotoxicity by Inhibiting HER-2/neu-Associated Antiapoptotic Signals. J. Cell. Biochem., In press.

(4) Ki M. Kim and Yong J. Lee: Pretreatment of acetyl salicylic acid promotes TRAIL-induced apoptosis by downregulating Bcl-2 gene expression. Manuscript in preparation.

Conclusions

Aspirin, an inhibitor of the PI3K-Akt-NF- B signal transduction pathway, enhances TRAIL-induced cytotoxicity *in vitro*. Therefore, aspirin may also be a useful drug to promote TRAIL cytotoxicity in HER-2/neu overexpressing prostate tumor cells.

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Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the P13K-Akt pathway

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We have previously shown that low extracellular pH (pHe) promotes cell killing by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In this study, we examined whether amiloride, an inhibitor of the Na^+/H^+ antiporter capable of lowering the intracellular pH (pHi), can potentiate TRAIL-induced apoptotic death. Human prostate adenocarcinoma DU-145 cells were treated with various concentrations of TRAIL (10–200 ng/ml) and/or amiloride (0.1–1 mM) for 4 h. Amiloride, which caused little or no cytotoxicity by itself, enhanced TRAIL-induced apoptosis. The TRAIL-mediated activation of caspase, and PARP (poly (ADP-ribose) polymerase) cleavage were both promoted by amiloride. Western blot analysis showed that combined treatment with TRAIL and amiloride did not change the levels of TRAIL receptors (death receptor (DR)4, DR5, and DcR2 (decoy recepter 2) or antiapoptotic proteins (FLICE-inhibitory protein (FLIP), inhibitor of apoptosis (IAP), and Bcl-2). However, unlike pHe, amiloride promoted the dephosphorylation of Akt. Interestingly, amiloride also induced the dephosphorylation of P13K (phosphatidylinositol 3-kinase) and PDK-1 (phosphoinositide-dependent kinase-1) kinases along with PTEN (phosphatase and tensin homolog deleted on chromosome 10) and PP1α phosphatases. In vitro kinase assays revealed that amiloride inhibited phosphorylation of kinases and phosphatases by competing with ATP. Taken together, the present studies suggest that amiloride enhances TRAIL-induced cytotoxicity by inhibiting phosphorylation of the PI3K-Akt pathway-associated kinases and phosphatases.

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Introduction

The abnormalities of the tumor vasculature, that is, a loss of the natural hierarchy of blood vessels, changes in the vascular density, and loss of the physiological regulation of blood perfusion, have been well documented (Endrich et al., 1982). These abnormalities cause an insufficient blood supply and development of a pathophysiological tumor microenvironment including low extracellular pH (pHe) (Wike-Hooley et al., 1984). Recently, we demonstrated that low pHe augments apoptosis induction by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a potent anticancer agent (Lee et al., 2004). The effect of a low pH is probably due to cytosol acidification and loss of the H^+ gradient normally presents across the inner membrane of mitochondria during apoptosis. TRAIL-induced association of truncated Bid with Bax and cytochrome c release are facilitated at low pH (Lee et al., 2004).

In this study, we investigated whether amiloride (3,5 diamino-6-chloro-N-(diaminomethylene) pyrazinecarboximide), an inhibitor of the Na^+/H^+ antiporter, can promote TRAIL-induced apoptotic death. It is well known that mammalian cells possess effective intracellular pH (pHi) regulatory mechanisms, such as the Na^+/H^+ antiporter and HCO_3^-/Cl^- exchange, through which the intracellular environment is maintained at a near neutral pH even in an acidic extracellular environment (Frelin et al., 1988). We postulate that acidification of the pHi by blocking the Na^+/H^+ antiporter enhances TRAIL cytotoxicity.

TRAIL/APO-2L is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family. TRAIL is a 281-amino-acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand (FasL) and TNF, the C-terminal extracellular region of TRAIL (amino acids 114–281) exhibits a homotrimeric subunit structure (Pitti et al., 1996). TRAIL binds to the death receptors such as TRAIL-R1 (DR4, death recepter 4) and TRAIL-R2 (DR5, death recepter 5), and induces the apoptotic signal. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1, decoy recepter 1) and TRAIL-R4 (DcR2, decoy recepter 2), which act as decoy receptors by inhibiting TRAIL signaling (Degli-Esposti et al.,

1997a, b; Marsters *et al.*, 1997; Pan *et al.*, 1997a, b; Sheridan *et al.*, 1997; Walczak *et al.*, 1997). Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain, while DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif (Pan *et al.*, 1997a, b). The relative resistance of normal cells to the apoptotic-inducing effects of TRAIL has been explained by the presence of large numbers of the decoy receptors on normal cells (Gura, 1997; Ashkenazi and Dixit, 1999).

Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell line (Keane *et al.*, 1999) and melanoma cell lines (Griffith *et al.*, 1998). This discrepancy indicates that other factors such as death inhibitors including FLICE (Fas-associated death domain-like interleukin-1β-converting enzyme)-inhibitory protein (FLIP) (Griffith *et al.*, 1998), Fas-associated protein (FAP-1) (Sato *et al.*, 1995), Bcl-2 (Wen *et al.*, 2000), Bcl-X$_L$ (Wen *et al.*, 2000), Bruton's tyrosine kinase (BTK) (Vassilev *et al.*, 1999), silencer of death domain (SODD) (Tschopp *et al.*, 1999), toso (Hitoshi *et al.*, 1998), inhibitor of apoptosis (IAP) (Kothny-Wilkes *et al.*, 1999), X-linked inhibitor of apoptosis (XIAP) (Deveraux and Reed, 1999), and survivin (Tamm *et al.*, 1998) may be responsible for the differential apoptotic effect of TRAIL. Previous studies show that chemotherapeutic agents (Griffith *et al.*, 1998; Keane *et al.*, 1999; Nagane *et al.*, 2000) and ionizing radiation (Chinnaiyan *et al.*, 2000) can increase TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP (Griffith *et al.*, 1998) or increasing DR5 gene expression (Sheikh *et al.*, 1998; Chinnaiyan *et al.*, 2000; Nagane *et al.*, 2000). We hypothesize that amiloride promotes TRAIL-induced apoptotic death by modulating the levels of TRAIL receptors and antiapoptotic molecules.

In addition to alterations of the levels of TRAIL receptors and antiapoptotic molecules, post-translational modification of antiapoptotic molecules such as phosphorylation of Akt may contribute to amiloride's potentiation of TRAIL-induced apoptosis. Akt (also known as PKB) is a serine/threonine kinase. It was originally identified as the cellular counterpart of the v-Akt transforming protein of a retrovirus (AKT8) that caused T-cell lymphomas in mice (Staal, 1987). Akt is recruited to the plasma membrane and activated by phosphorylation at threonine 308 and serine 473 in response to growth factors (Alessi *et al.*, 1996). The molecule responsible for the recruitment of Akt is phosphatidylinositol (PI) 3-kinase (PI3K) (Alessi *et al.*, 1996; Andjelkovic *et al.*, 1997). PI3K consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, activating its catalytic subunit (P110) (Rodriguez-Viciana *et al.*, 1996). PI3K phosphorylates phosphoinositides at the 3′-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (PIP$_3$) (Rameh and Cantley, 1999). PIP$_3$ facilitates the recruitment of Akt to the plasma

membrane through binding with the pleckstrin homology (PH) domain of Akt (Rameh and Cantley, 1999). At the plasma membrane, Akt is activated by phosphoinositide-dependent kinase-1 (PDK-1) through phosphorylation at threonine 308 and serine 473 (Anderson *et al.*, 1998; Andjelkovic *et al.*, 1997). Activated Akt phosphorylates several transcription factors (e.g. NF-κB, Forkhead, CREB) (Du and Montminy, 1998; Romashkova and Makarov, 1999; Kashii *et al.*, 2000) as well as proapoptotic molecules such as Bad and procaspase-9. Phosphorylation of Bad and procaspase-9 results in inactivation of these molecules and inhibits apoptosis (Datta *et al.*, 1997; Kennedy *et al.*, 1997; Cardone *et al.*, 1998). In this study, we observed that amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of Akt rather than by decreasing the levels of TRAIL receptors or antiapoptotic molecules.
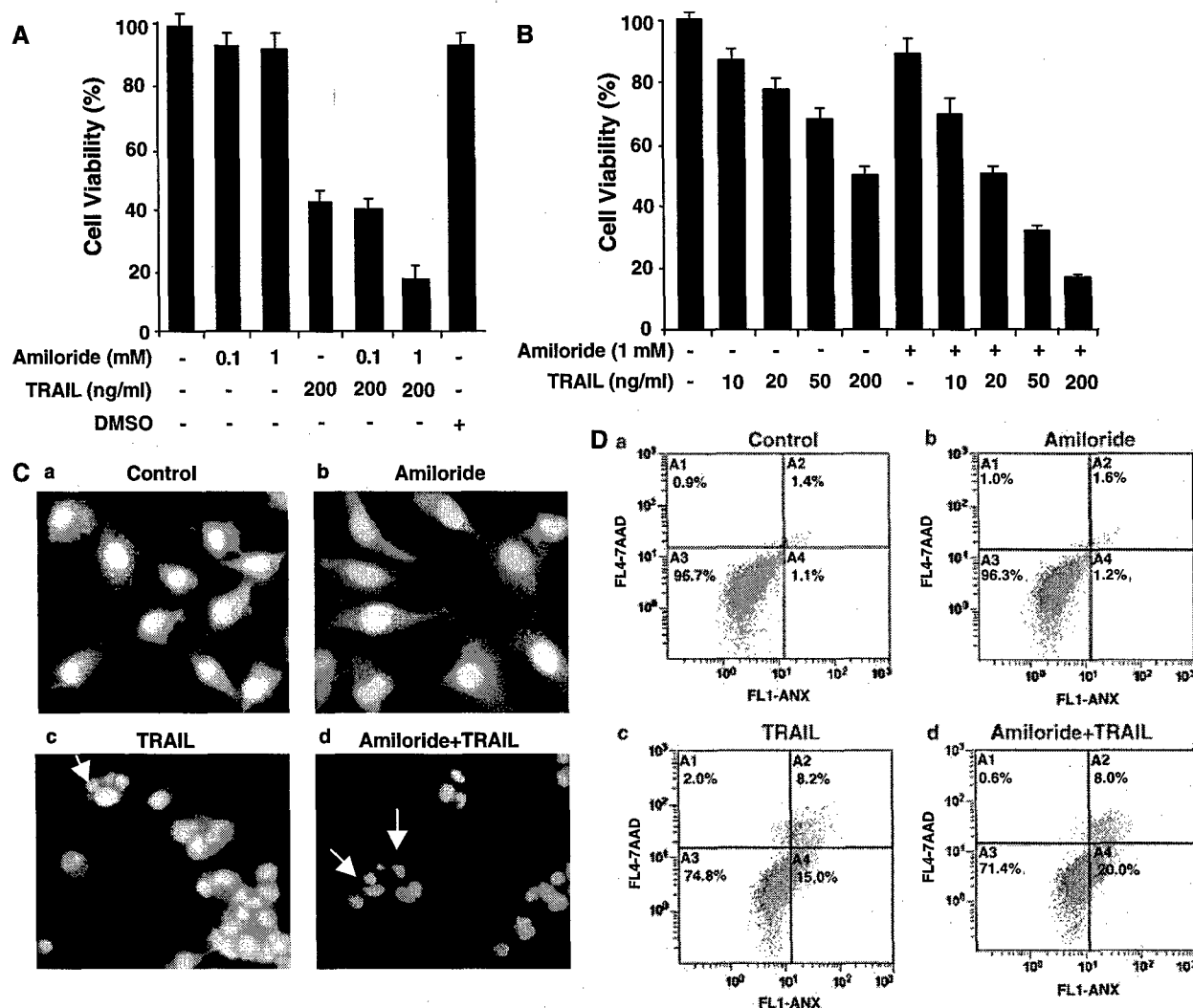
Results

Amiloride enhances TRAIL-induced cytotoxicity

To investigate the effect of amiloride on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma DU-145 cells were treated with TRAIL in the presence of absence of amiloride. Figure 1a and b show that little or no cytotoxicity was observed with amiloride (0.1–1 mM) alone. In contrast, TRAIL-induced cytotoxicity was concentration-dependent, and was significantly increased in the presence of 1 mM amiloride (Figure 1b). For example, when cells were treated for 4 h with 200 ng/ml TRAIL in the presence or absence of 1 mM amiloride, the surviving fraction was 17 or 43%, respectively. Similar results were observed by DAPI (4′,6-diamidino-2-phenylindole) staining (Figure 1c). DAPI staining of cells treated with TRAIL in combination with amiloride showed the presence of many cells with condensed nuclei, a morphological change that is associated with apoptosis. Apoptotic death was also evaluated by flow cytometric analysis in combination with 7-amino-actinomycin-D (7-ADD) and annexin V staining assays (Figure 1d). Figure 1d clearly shows that amiloride promoted TRAIL-induced apoptotic death (annexin V + /7-AAD−).

Effect of amiloride on TRAIL-induced apoptosis

Additional studies were designed to examine whether the combination of amiloride and TRAIL treatment of DU-145 cells enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis. PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL (Figure 2). This cleavage was enhanced by treatment with amiloride (Figure 2). For example, data from densitometer analysis show that amiloride promoted a 3.6-fold increase in the 85 kDa fragment when treatment of cells with 10 ng/ml TRAIL in combination with amiloride was compared with TRAIL alone. The cleavage of

Figure 1 Effect of amiloride on TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells. (A) Cells were treated for 4 h with 200 ng/ml TRAIL in the presence or absence of amiloride (0.1–1 mM). (B) Cells were treated for 4 h with various concentrations of TRAIL (0–200 ng/ml) in the presence or absence of 1 mM amiloride. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (s.e.m.) from three separate experiments. (C) Cells were treated for 4 h with TRAIL (200ng/ml) in the presence or absence of 1 mM amiloride. After treatment, cell were stained with DAPI (1 μg/ml), and morphological features were analysed with a fluorescence microscope. Nuclei of apoptotic cells are fragmented and condensed, indicated by arrows. (D) Cells were treated with 10 ng/ml TRAIL for 4 h in the presence or absence of 1 mM amiloride. After treatment, cells were stained with FITC-annexin V and 7-ADD, and then analysed with a FACScan flow cytometer

PARP was not observed by treatment with amiloride alone (data not shown). We extended our studies to investigate whether amiloride enhances TRAIL-induced cytotoxicity by increasing the activation of caspase. Figure 2 demonstrates that amiloride promoted TRAIL-induced caspase-8 activation. Western blot analysis shows that procaspase-8 (54/55 kDa) was cleaved to the intermediates (41 and 43 kDa) and active form (18 kDa) in the presence of TRAIL. The cleavage of procaspase-8 was promoted by treatment with amiloride. The combined treatment of TRAIL and amiloride also resulted in an increase in caspase-9 activation (Figure 2). TRAIL induced proteolytic processing of procaspase-9 (48 kDa) into its active form (37 kDa). The

activation of caspase-9 induced by TRAIL was enhanced by amiloride (Figure 2). Amiloride also increased TRAIL-induced caspase-3 activation (Figure 2). Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) in the presence of TRAIL. The combined treatment with amiloride and TRAIL increased the level of the active form. Amiloride alone did not activate caspases (data not shown). We further examined whether amiloride affects apoptosis by a stimulus other than TRAIL. Like TRAIL, FasL induced PARP cleavage, the hallmark feature of apoptosis (Figure 3a). This cleavage was promoted by treatment with amiloride (lane 3 versus lane 5 in Figure 3a). We also examined

TRAIL (ng/ml)   -   10   20   50   200   10   20   50   200
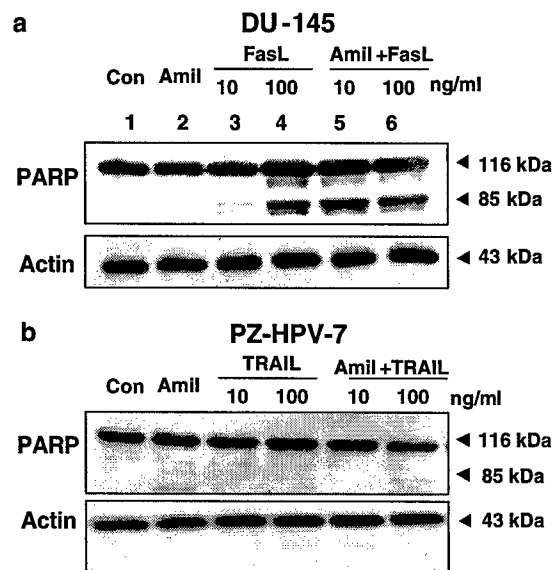Amiloride (mM)   -   -   -   -   -   1   1   1   1
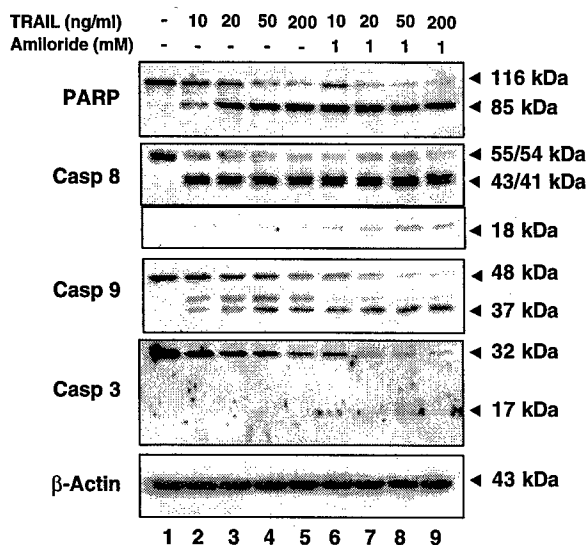
Figure 2 Effect of amiloride on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in DU-145 cells. Cells were treated for 4 h with various concentrations of TRIL (0–200 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. Cell lysates were subjected to immunoblotting for caspase-8, caspase-9, caspase-3, or PARP. Antibody against caspase-8 detects inactive from (55/54 kDa), and cleaved intermediates (41, 43 kDa). Anti-caspase-9 antibody detects both inactive from (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane
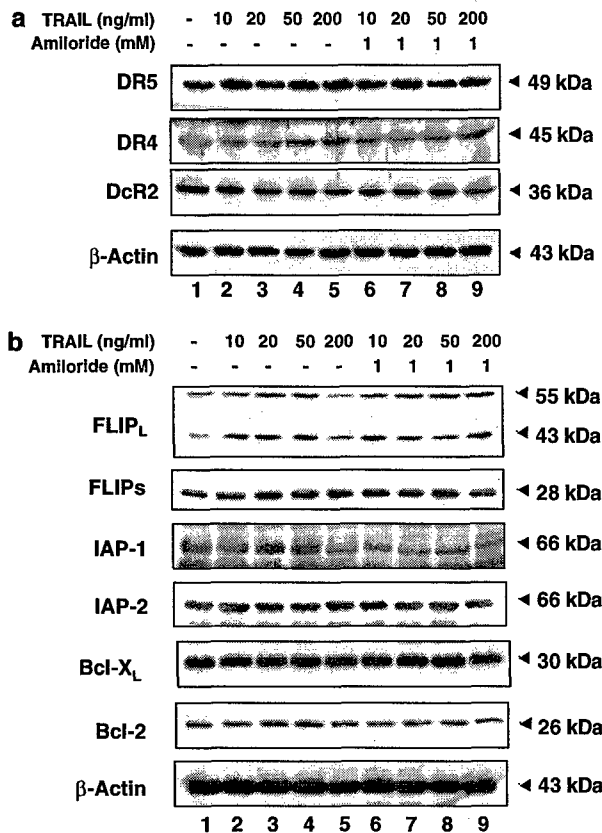

Figure 3 Effect of amiloride on Fas Ligand (FasL) or TRAIL-induced proteolytic cleavage of PARP in DU-145 or PZ-HPV7 cells, respectively. (a) DU-145 cells were treated for 4 h with various concentrations of FasL (10–100 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. (b) PZ-HPV7 cells were treated for 4 h with various concentrations of TRAIL (10–100 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. Cell lysates were subjected to immunoblotting for PARP as described in Figure 2. Actin was shown as an internal standard

whether amiloride enhances TRAIL-induced apoptosis in normal prostate epithelial PZ-HPV-7 cells. Unlike DU-145 cells, neither TRAIL alone nor the combined treatment of TRAIL and amiloride induced PARP cleavage in PZ-HPV-7 cells (Figure 3b).

Effects of amiloride on the level of TRAIL receptor family and antiapoptotic proteins

Previous studies demonstrate that increased DR5 levels induced by genotoxic agents (Sheikh et al., 1998; Chinnaiyan et al., 2000; Nagane et al., 2000) or decreased FLIP expression induced by glucose deprivation (Nam et al., 2002) is responsible for increasing TRAIL cytotoxicity. Thus, we examined whether changes in the amounts of TRAIL receptors and antiapoptotic proteins are associated with the promotion of apoptosis by TRAIL in combination with amiloride. DU-145 cells were treated with 200 ng/ml TRAIL in the presence of 1 mM amiloride. Data from Western blot analysis reveal that the combined treatment did not significantly alter the levels of DR4, DR5, DcR2, $FLIP_L$, $FLIP_S$, IAP-1, IAP-2, $Bcl-X_L$, and Bcl-2 (Figure 4). Amiloride alone also did not change the levels of TRAIL receptors and antiapoptotic proteins (data not shown).

Effect of amiloride on Akt phosphorylation

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis (Nesterov et al., 2001). We postulated that amiloride inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity. To examine whether amiloride inhibits Akt activity by dephosphorylating Akt, DU-145 cells were treated with 1 mM amiloride for various periods (5–60 min) and the level of phosphorylated Akt was measured. Figure 5a shows that Akt was rapidly dephosphorylated within 5 min of amiloride addition without changing the Akt protein level. Data from densitometer analysis show a 16.3-fold decrease in the level of phosphorylated Akt during treatment with amiloride. TRAIL treatment did not alter amiloride-induced dephosphorylation of Akt (Figure 5b). Since several researchers have demonstrated that overexpression of Na^+/H^+ exchanger promotes cell survival and Akt activity (Barriere et al., 2001; Wu et al., 2004), we investigated whether another ion transport inhibitor such as DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) dephosphorylates Akt. Figure 5c shows that Akt was also dephosphorylatd by treatment with 200 μM DIDS. These results suggest that promoting intracellular acidification inhibits Akt activity by dephosphorylation. Thus, we further investigated whether pHe also dephosphorylates Akt. Figure 5d reveals that pHe did not alter the level of phosphorylated Akt. These results suggests that the mechanism of pHe-enhanced

a TRAIL (ng/ml) - 10 20 50 200 10 20 50 200
Amiloride (mM) - - - - - 1 1 1 1

DR5 ◄ 49 kDa

DR4 ◄ 45 kDa

DcR2 ◄ 36 kDa

β-Actin ◄ 43 kDa

1 2 3 4 5 6 7 8 9

b TRAIL (ng/ml) - 10 20 50 200 10 20 50 200
Amiloride (mM) - - - - - 1 1 1 1

FLIPL ◄ 55 kDa
◄ 43 kDa

FLIPs ◄ 28 kDa

IAP-1 ◄ 66 kDa

IAP-2 ◄ 66 kDa

Bcl-XL ◄ 30 kDa

Bcl-2 ◄ 26 kDa
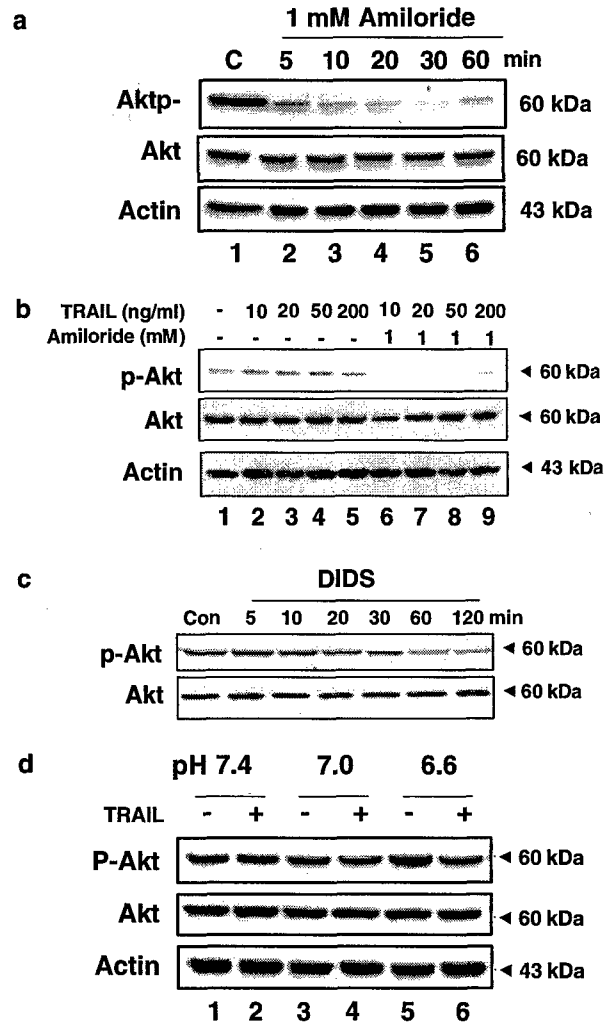
β-Actin ◄ 43 kDa

1 2 3 4 5 6 7 8 9

Figure 4 Intracellular levels of TRAIL receptors (a) or anti-apoptotic proteins (b) during treatment with TRAIL in the presence or absence of amiloride. DU-145 cells were treated for 4 h with various concentrations of TRAIL (0–200 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted as described in Materials and methods. Actin was shown as an internal standard
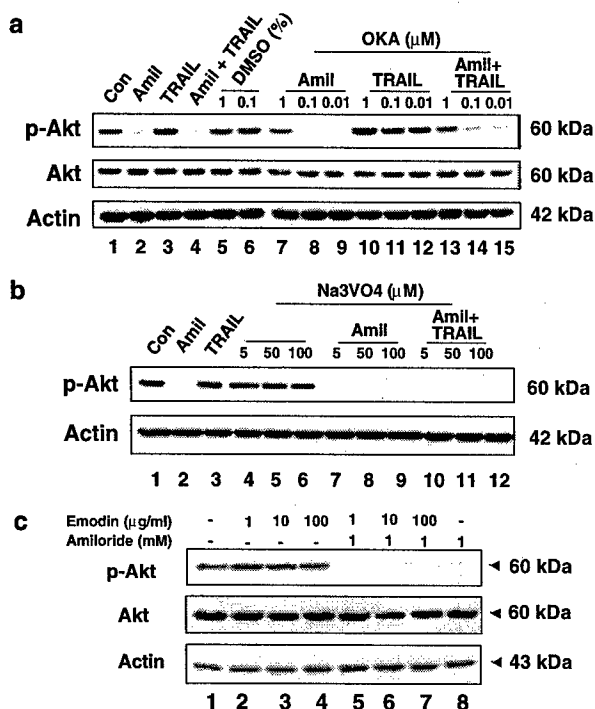
a 1 mM Amiloride
C 5 10 20 30 60 min

Aktp- 60 kDa

Akt 60 kDa

Actin 43 kDa

1 2 3 4 5 6

b TRAIL (ng/ml) - 10 20 50 200 10 20 50 200
Amiloride (mM) - - - - - 1 1 1 1

p-Akt ◄ 60 kDa

Akt ◄ 60 kDa

Actin ◄ 43 kDa

1 2 3 4 5 6 7 8 9

c DIDS
Con 5 10 20 30 60 120 min

p-Akt ◄ 60 kDa

Akt ◄ 60 kDa

d pH 7.4 7.0 6.6

TRAIL - + - + - +

P-Akt ◄ 60 kDa

Akt ◄ 60 kDa

Actin ◄ 43 kDa

1 2 3 4 5 6

Figure 5 Effect of amiloride (a and b), DIDS (c), or pHe (d) on the levels of phosphorylated Akt in DU-145 cells. (a) Cells were treated with 1 mM amiloride for various times (0–60 min) and then harvested. (b) Cells were treated for 4 h with various concentrations of TRAIL (0–200 ng/ml) in the presence or absence of 1 mM amiloride and then harvested. (c) Cells were treated with 200 μM DIDS for various times (0–120 min) and then harvested. (d) Cells were treated with TRAIL (200 ng/ml) or without TRAIL (0 ng/ml) for 4 h at various extracellular pH (6.6–7.4) and then harvested. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody. Actin was shown as an internal standard

TRAIL cytotoxicity (Lee et al., 2004) is not the same as that of amiloride-promoted TRAIL apoptotic death.

To examine whether dephosphorylation of Akt during amiloride treatment is mediated through activation of phosphatase, we treated DU-145 cells with okadaic acid, a serine phosphatase inhibitor. Figure 6a shows that amiloride-induced Akt dephosphorylation was suppressed by pretreatment with 1 μM, but not by 0.1 μM, okadaic acid. For example, data from densitometer analysis show that the level of phosphorylated Akt was reduced by 94% during treatment with amiloride. However, the phosphorylated Akt level decreased only 13% in the 1 μM okadiac acid-pretreated cells. It is well known that okadaic acid inhibits both protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) both serine/threonine phosphatases, at higher concentrations (IC$_{50}$ = 150 nM), but it inhibits only PP2A at low concentrations (IC$_{50}$ < 0.1 nM). These results suggest that PP1 rather than PP2A plays an important role in the regulation of Akt phosphorylation, since recent studies have demonstrated that dephosphorylation of Akt is regulated by PP1 (Xu et al.,

2003). Unlike okadaic acid, sodium orthovanadate (Na$_3$VO$_4$), an inhibitor of tyrosin phosphatases, and emodin, a tyrosine kinase inhibitor, did not prevent dephosphorylation of Akt during treatment with amiloride (Figure 6b and c). As a next step, we repeated the procedure to examine a possible involvement of PP1 in the amiloride-induced dephosphorylation of Akt. Figure 7a shows that amiloride induced dephosphorylation (activated) of PP1 and 1 μM okadaic acid substantially prevented dephosphorylation of PP1. Amiloride-promoted TRAIL-induced PARP cleavage was also significantly inhibited by treatment with 1 μM okadaic acid (Figure 7b).
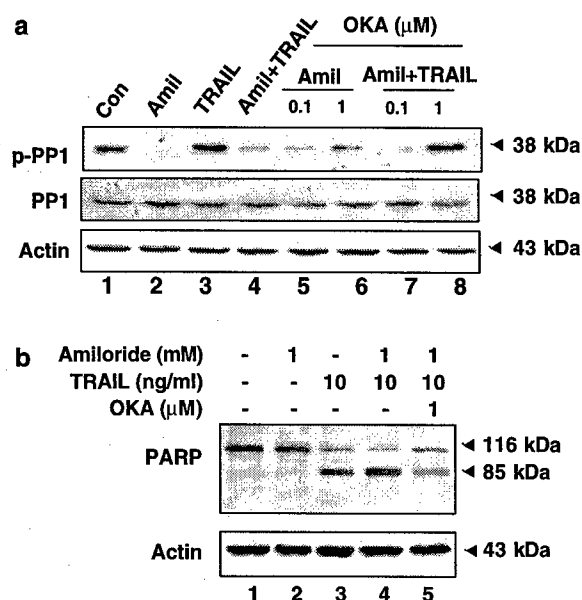
Figure 6 Effect of okadaic acid, sodium orthovanadate, or emodin on amiloride-induced dephosphorylation of Akt. (a) DU-145 cells were pretreated with okadaic acid (0.01–1 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (b) Cells were pretreated with sodium orthovanadate (5–100 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (c) Cells were treated for 1 h with various concentrations of emodin (1–100 μg/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody. Actin was shown as an internal standard


Figure 7 Effect of okadaic acid on amiloride-induced PP1 dephosphorylation (a) or TRAIL in combination with amiloride-induced PARP cleavage (b). (a) DU-145 cells were pretreated with okadaic acid (0.1–1 μM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride. (b) Cells were pretreated with 1 μM okadaic acid for 30 min and treated with TRAIL in the presence or absence of amiloride. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho-PP1, anti-PP1, or anti-PARP antibody. Actin was shown as an internal standard

Effect of amiloride on kinases and phosphatases associated with the PI3K-Akt pathway

Previous studies demonstrated that Akt activation is regulated through the PI3K-Akt pathway. We further examined whether amiloride specifically activates PP1 activity or nonspecifically affects the PI3K-Akt pathway-associated kinases and phosphatases. Figure 8a shows that amiloride induced dephosphorylation of not only PP1 but also PTEN (phosphatase and tensin homolog deleted on chromosome 10), which is known to be a major negative regulator of the PI3K-Akt signaling pathway. Amiloride also induced dephosphorylation of the PI3K-Akt pathway-associated kinases such as PI3K and PDK-1 (Figure 8b). We further examined whether amiloride had an affect on phosphorylation of a substrate that is not in the PI3K/PDK-1/Akt signaling pathway. Figure 8c shows that hydrogen peroxide activated JNK (phosphorylation of JNK1 and JNK2) and its phosphorylation was not inhibited by treatment with amiloride.

The effect of amiloride on Akt was compared with LY294002 and wortmannin, inhibitors of PI3K. Figure 9a shows that all these drugs induced dephos-

phorylation of Akt. However, unlike amiloride, LY294002 and wortmannin did not induce dephosphorylation of PI3K, PDK-1 and PTEN. Nevertheless, LY294002 and wortmannin promoted TRAIL-induced cytotoxicity (Figure 9b). These results indicate that Akt inactivation (dephosphorylation) is responsible for the amiloride-induced enhancement of TRAIL cytotoxicity.

Mechanism of amiloride-induced alterations of phosphatase and kinase activities

Davis and Czech (1985) reported that amiloride acts as an ATP analog that causes the formation of nonproductive enzyme–substrate complexes. We hypothesized that amiloride competes with ATP, thereby inhibiting protein posphorylation. To test this hypothesis, as a first step we investigated whether amiloride directly dephosphorylates an active Akt. Figure 10 shows that amiloride did not directly dephosphorlylate Akt when mixed with phosphorylated Akt (lane 2 in Figure 10). In addition, amiloride did not inhibit dephosphorylation of Akt by PP1 (lane 5 in Figure 10). As a next step, we examined whether amiloride blocks the kinase-mediated phosphorylaton process. Figure 11a shows that unphosphorylated Akt was phosphorylated by active PDK-1 in vitro. Amiloride, but not dimethylsulfoxide (DMSO), inhibited phosphorylation of Akt (Figure 11b). Data from densitometer analysis show that phosphorylation
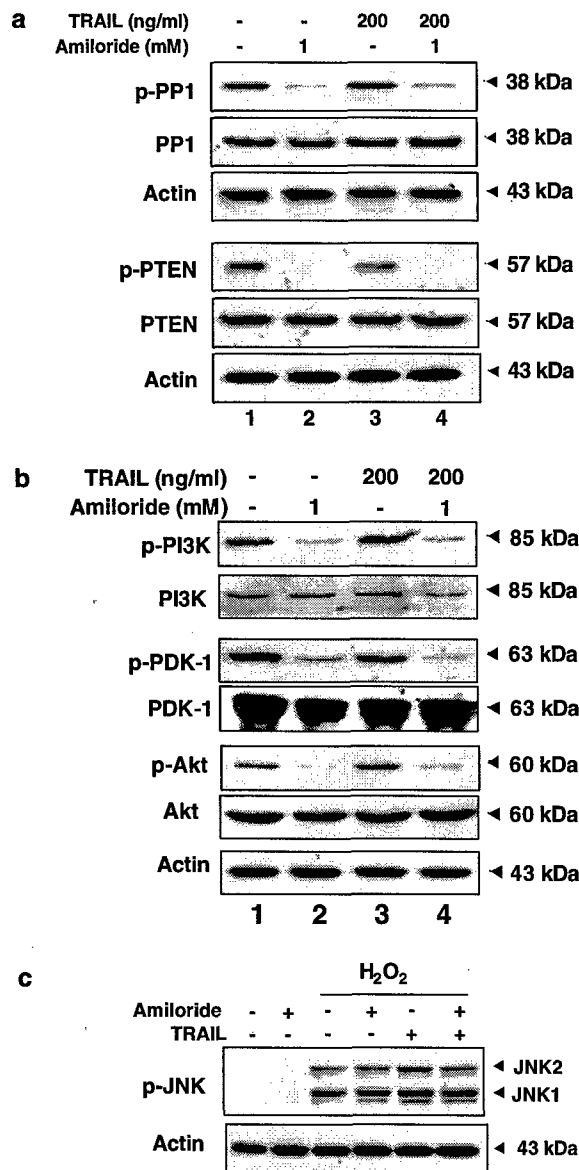
Figure 8 Effect of amiloride on PI3K-Akt pathway-associated phosphatases (a) and kinases (b), or JNK (c) in the presence or absence of TRAIL. (a and b) DU-145 cells were treated for 2 h with 1 mM amiloride in the presence or absence of 200 ng/ml TRAIL. (c) Cells were treated with H_2O_2 (200 μM) for 1 h in the presence or absence of TRAIL (200 ng/ml)/amiloride (1 mM) and then harvested. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in Materials and methods. Actin was shown as an internal standard

Figure 9 Effect of LY294002 or wortmannin on kinases/phosphatases (a) and TRAIL-induced cytotoxicity (b) in DU-145 cells. (a) Cells were treated for 1 h with LY294002 (2–20 μM), wortmannin (20–200 nM), or 1 mM amiloride. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in Materials and methods. Actin was shown as an internal standard. (b) Cells were pretreated with 20 μM LY294002 or 200 nM wortmannin for 20 min and then treated or not treated with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (s.e.m.) from three separate experiments

of Akt was decreased by 87% during treatment with amiloride. Similar results are shown in Figure 12. Casein kinase II (CK2), a protein kinase for PTEN, phosphorylated PTEN *in vitro* and its phosphorylation was blocked by amiloride in a dose-dependent manner (Figure 12a). Figure 12b shows that amiloride blocks the kinase-mediated phosphorylaton process by competing with ATP.

Discussion

Amiloride is known to inhibit tyrosine kinase activity of growth factor receptors (Davis and Czech, 1985), Na^+/Ca^{2+} exchange (Smith *et al.*, 1982), (Na^+-K^+)-ATPase (Soltoff and Mandel, 1983), and serine kinase activity (Ralph *et al.*, 1982). The data presented in this report demonstrate that amiloride is a potent promoter of TRAIL-induced apoptotic death (Figures 1 and 2). This is probably due to the amiloride-induced dephosphorylation (inactivation) of Akt, an antiapoptotic protein. Our studies also reveal that amiloride causes a decrease

Active PP1α	-	-	-	+	+	+
p-Akt	+	+	+	+	+	+
Amiloride (1mM)	-	+	-	-	+	-
DMSO(1%)	-	-	+	-	-	+

p-Akt ◄ 60 kDa

Akt ◄ 60 kDa

1 2 3 4 5 6
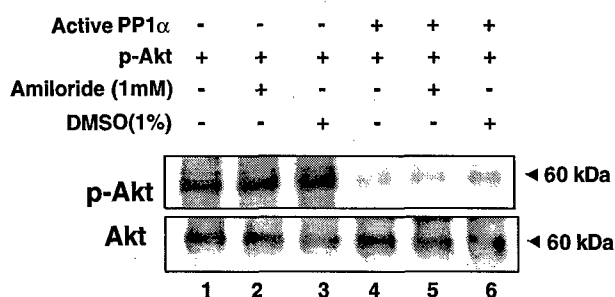
Figure 10 Effect of amiloride on phosphorylated Akt protein or PP1-mediated dephosphorylation of Akt. Purified active Akt protein was incubated with PP1, amiloride, or DMSO. The sample was separated by SDS–PAGE and immunoblotted with anti-phospho-Akt or anti-Akt antibody

a

myc-PDK-1	-	+
Akt	+	+

WB: p-Akt ◄ 60 kDa

WB: Akt ◄ 60 kDa

WB: PDK-1 ◄ 63 kDa

IP : myc

b

myc-PDK-1	+	+	+
Akt	+	+	+
Amiloride (1mM)	-	+	-
DMSO (1%)	-	-	+

WB:p-Akt ◄ 60 kDa

WB: Akt ◄ 60 kDa

WB: PDK-1 ◄ 63 kDa

IP : myc

WB: Actin ◄ 43 kDa

Cell lysate
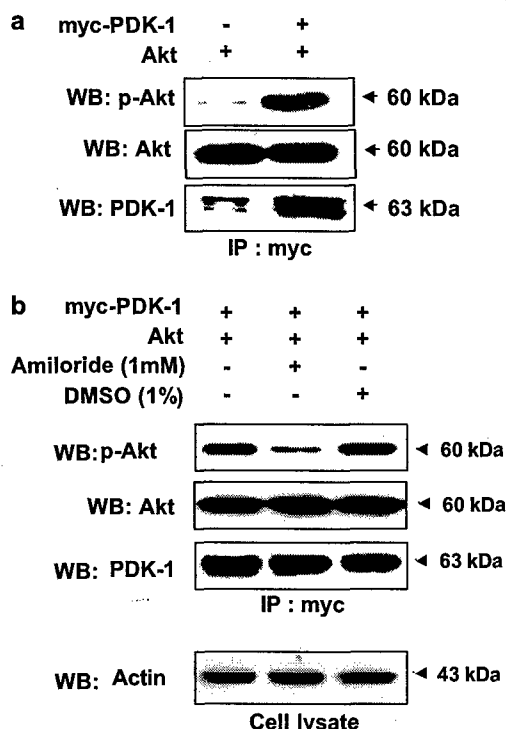
Figure 11 Effect of amiloride on PDK-1 kinase activity. DU-145 cells were transfected with pcDNA3myc-PDK-1. At 1 day after transfection, myc-PDK-1 was immunoprecipitated with anti-myc antibody. For in vitro PDK-1 kinase assays, the immune complex was incubated with purified inactive Akt protein in the presence or absence of amiloride. The sample was separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, anti-PDK-1 antibody

in the phosphorylation state of Akt by inhibiting (dephosphorylation of) PI3K and PDK-1 kinases as well as activating (dephosphorylation of) PTEN and PP1 (Figure 8). The inhibition of kinases by amiloride can be relieved at high ATP concentrations which indicates that the inhibition of kinase activity is competitive with ATP (Figure 12b; Holland et al., 1983). Thus, amiloride may act as an ATP analog and directly inhibit kinase activity by decreasing protein

phosphorylation (Davis and Czech, 1985). Indeed, our results from in vitro kinase assays show that amiloride directly inhibits PDK-1 and CK2 kinase activity (Figures 11 and 12).

It has previously been suggested that a possible mechanism of biochemical action of amiloride is the inhibition of kinase activity (Davis and Czech, 1985; Ralph et al., 1982). In addition to the inhibition of kinase activity, our data indicate that amiloride activates PTEN and PP1 phosphatases. PTEN protein is composed of an N-terminal dual specificity phosphatase-like enzyme domain, and a C-terminal regulatory domain that binds to phospholipid membranes. It exhibits phosphatase activity towards both protein substrates and the lipid second messenger, PIP3 (Maehama and Dixon, 1998; Myers et al., 1997). PTEN reverses the action of PI3K by catalyzing the removal of the 3' phosphate of PIP3. PTEN is phosphorylated by protein kinase CK2 (Torres and Pulido, 2001) in a constitutive manner at a cluster of Ser/Thr residues located in the C-terminal tail region. The C-terminal tail region also contains the PSD-95/Dlg/ZO-1 homology (PDZ) domain-binding sequence. Recent studies reveal that phosphorylation of the C-terminal tail targets PTEN to the plasma membrane, not by blocking the PDZ domain-binding site but by interfering with the electrostatic membrane binding of PTEN (Das et al., 2003). In this study, we observed that PTEN was dephosphorylated (activated) by treatment with amiloride (Figure 8a), causing it to bind to the plasma membrane. This suggests that the plasma membrane bound PTEN mediates the amiloride-induced dephosphorylation (inactivation) of Akt by inhibiting recruitment of Akt to the plasma membrane and consequently enhancing TRAIL cytotoxicity (Kandasamy and Srivastava, 2002). Previous studies showed that Akt activity was also regulated by Ser/Thr phosphatases such as PP2A (Resjo et al., 2002) or PP1 (Xu et al., 2003). However, pharmacokinetics studies reveal that PP1 rather than PP2A is responsible for regulation of Akt phosphorylation (Xu et al., 2003). It is well known that the activity of PP1 is regulated by phosphorylation of its catalytic subunit. Upon phosphorylaton of Thr^{320}, the COOH terminus of PP1 folds back to mask its catalytic center (Goldberg et al., 1995). Our data clearly demonstrate that amiloride dephosphorylates (activates) PP1 (Figure 7) as well as Akt (Figures 5 and 6). The amiloride-mediated dephosphorylation of PP1 and Akt is inhibited by 1 μM, but not by 0.1 μM of okadaic acid (Figure 7). Previous pharmacokinetics studies demonstrate that okadaic acid inhibits only PP2A at low concentrations (IC$_{50}$ < 0.1 nM), but both PP1 and PP2A at higher concentrations (IC$_{50}$ = 150 nM). Taken together, our results are consistent with previous observations that PP1 rather than PP2A regulates Akt phosphorylation (Xu et al., 2003).

Previous studies have shown that constitutively active Akt blocks TRAIL cytotoxicity (Chen et al., 2001; Thakkar et al., 2001). Downregulation of Akt activity of PI3K inhibitors, wortmannin and LY294002, promotes TRAIL cytotoxicity (Figure 9; Thakkar et al., 2001;
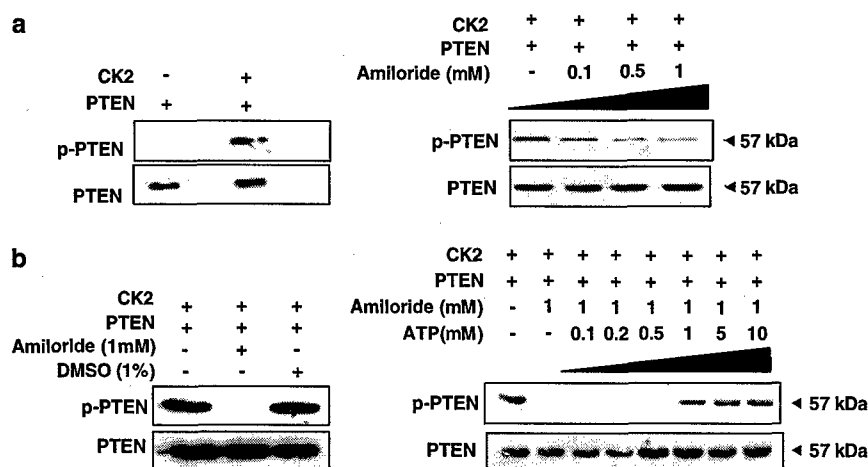
Figure 12 Effect of amiloride on CK2 kinase activity. For *in vitro* CK2 kinase assays, purified active PTEN protein was incubated with active CK2 in the presence or absence of various concentrations of amiloride (a) or various concentrations of ATP (b). The sample was separated by SDS–PAGE and immunoblotted with anti-phospho-PTEN or anti-PTEN antibody

Martelli *et al.*, 2003). This is probably due to inhibiting Akt-mediated antiapoptotic effects. A number of proapoptotic proteins have been identified as direct Akt substrates, including BAD, caspasase-9, and Fork-head transcription factors (Cross *et al.*, 1995; Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998; Brunet *et al.*, 1999; Hetman *et al.*, 2000). The proapoptotic function of these molecules is suppressed upon phosphorylation by Akt. Akt also induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB (Ozes *et al.*, 1999). Recently, we (Nam *et al.*, 2002) and Panka *et al.* (2001) reported that the PI3K-Akt-NF-κB pathway may regulate the expression of FLICE-inhibitory protein (FLIP), an antiapoptotic molecule. We postulate that amiloride-mediated depho-sphorylation of Akt leads to activation of proapoptotic molecules and results in enhancement of TRAIL-induced apoptotic death. Overall, our model may provide important insights into how amiloride promotes TRAIL-induced apoptotic death. We believe that this model provides a framework for future studies.

Materials and methods

Cell culture and survival assay

Human prostate adenocarcinoma DU-145 cell line and human normal prostate epithelial PZ-HPV-7 cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). DU-145 cells were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 26 mM sodium bicarbonate for monolayer cell culture. PZ-HPV-7 cells were cultured in keratinocyte serum-free medium (K-SFM, Gibco BRL) containing 10% fetal bovine serum, L-glutamine, human recombinant epidermal growth factor (5 ng/ml), and bovine pituitary extract (50 μg/ml). The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air

and 5% CO_2. At 1 day prior to the experiment, cells were plated into 60-mm dishes. For trypan blue exclusion assay (Burow *et al.*, 1998), trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light micro-scope. At least 300 cells were counted for each survival determination.

Drug treatment

Wortmanin, sodium orthovanadate, hydrogen peroxide, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), and 3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboximide (amiloride) were obtained from Sigma Chemical Co. (St Louis, MO, USA). LY294002 was purchased from Cell Signaling (Beverly, MA, USA), and okadaic acid from Calbiochem (San Diego, CA, USA). A stock solution was prepared in DMSO.

Production of recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT–PCR was cloned into a pET-23d (Novagen, Madison, WI, USA) plasmid, and His-tagged TRAIL protein was purified using the Qiagen express protein purification system (Qiagen, Valencia, CA, USA).

Morphological evaluation

Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome 4',6-diamidino-2-phenylindole (DAPI). Cells were grown on glass coverslips at a density of 2×10^5 cells/well. Cells treated with TRAIL, amiloride, or TRAIL in combination with amiloride for 4 h. Cells were washed twice with PBS and fixed by incubation in 70% ethanol for 30 min. Following washes with PBS, cells were incubated in 1 μg/ml DAPI solution for 30 min in the dark. Coverslips were then washed with PBS and analysed by fluorescence microscopy.

Flow cytometry

Cells were pelleted, washed with PBS, and resuspended in 200 μl of fluorescein isothiocyanate (FITC)-annexin V (1 μM/ml). After 15 min at room temperature, cells were washed with PBS and resuspended in 100 μl of fluorescence-activated cell-sorting buffer (PBS, 1% BSA, and 0.1% sodium azide) containing 7-ADD (20 μg/100 μl). Cells were incubated for 15 min at 4°C in the dark. Analysis was performed using the FACScan flow cytometer (Beckman Coulter, Inc., Hialeah, FL, USA), and results were analysed with CelloQuest software (Becton Dickinson Immunocytometry Systmes, San Jose, CA, USA).

Antibodies

Polyclonal anti-phospho-PI3K, anti-Bcl-X_L, anti-caspase-3, and anti-caspase-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA), anti-DR5 and anti-DcR2 from StressGen (Victoria, BC, Canada), anti-DR4 from Upstate Biotechnology (Lake Placid, NY, USA), anti-ACTIVE JNK from Promega (Madison, WI, USA); anti-cIAP-1 and anti-cIAP-2 from R&D Systems (Minneapolis, MN, USA), anti-phospho-Akt, anti-Akt, anti-phospho-Bad from Cell Signaling, and anti-FLIP from Calbiochem. Monoclonal antibodies were purchased from the following companies: anti-PI3K from Santa Cruz, anti-caspase-8 from Upstate Biotechnology, anti-cytochrome c from PharMingen (San Diego, CA, USA), anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA, USA), and anti-Bcl-2 and anti-actin from ICN (Costa Mesa, CA, USA).

Protein extracts and PAGE

Cells were lysed with 1 × Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–polyacrylamide gels. SDS–PAGE analysis was performed according to Laemmli (1970) using a Hoefer gel apparatus.

Immunoblot analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights IL, USA). Quantitation of X-ray film was carried out by scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) using area integration.

In vitro enzyme assays

For *in vitro* PP1 phosphatase assays, 10 μl of active PP1α proteins (1 U/ml; Upstate Biotechnology) were incubated with 3 μl of active Akt (0.5 μg; Upstate Biotechnology) for 30 min at 30°C in 50 μl of buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 100 μM ATP, 150 mM KCl, 5 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM DTT).The reaction tubes contained either 1 mM amiloride or the same volume of DMSO.

The reaction was stopped by adding 2 × SDS sample buffer and heating at 100°C for 5 min. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analysed by anti-phospho Akt antibody (Cell Signaling).

For *in vitro* PDK-1 kinase assays, DU-145 cells were transiently transfected with PDK-1 constructs (pcDNA3myc-PDK-1; 2 μg of DNA/dish) using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA). At 1 day after transfection, cells were lysed with 500 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Trition X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl, 10 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma)). An aliquot of each lysate was immunoblotted to verify overexpression of PDK-1 constructs. Another aliquot was immunoprecipitated by incubation with mouse anti-myc antibody (9E10; Roche, Indianapolis, IN, USA) and protein G-Plus agarose (Gibco BRL) for 2 h at 4°C. Immune complexes were washed twice with buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 mM DTT, 1 mM Na$_3$VO$_4$, and 1 mM PMSF) at 4°C and then incubated with 2 μg of purified inactive Akt protein (Upstate Biotechnology) in a volume of 50 μl of a kinase buffer (100 μM ATP, 20 mM Tris-HCl (pH 7.5), 20 mM MgCl, 0.1 mM EDTA) for 30 min at 30°C. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analysed by rabbit anti-phospho-Akt antibody (Cell Signaling).

For *in vitro* casein kinase 2 (CK2) kinase assays, 12.5 μl of active PTEN proteins (0.5 μg; Upstate) were incubated with 5 μl of active CK2 (0.5 μg; Upstate) for 30 min at 30°C in 40 μl of kinase buffer (20 mM Tris-HCl, pH 7.5 5 mM MgCl$_2$, 200 μm ATP, 150 mM KCl, 5 mM β-glycerolphosphate, 0.1 mM Na$_3$VO$_4$, 2 mM DTT). The reaction was stopped by adding 2 × SDS sample buffer and heating at 100°C for 5 min. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of PTEN was analysed by anti-phospho PTEN antibody (Cell Signaling).

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Hypoxia and low glucose differentially augments TRAIL-induced apoptotic death

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Abstract

Tumor microenvironment, which is characterized by hypoxia, low-glucose concentrations, high-lactate concentrations, low-extracellular pH, can alter the therapeutic response in tumors. In this study, we investigated whether hypoxia affects TRAIL-induced apoptotic death. When human prostate adenocarcinoma DU-145 cells were treated with 50 ng/mL TRAIL or hypoxia for 4 h, the survival was 45.7 and 32.5%, respectively. The combination of TRAIL and hypoxia synergistically increased cell death. Similar results were observed in human prostate adenocarcinoma LNCaP cells. Western blot analysis showed that the hypoxia augmented TRAIL-induced PARP cleavage as well as the activation of caspase-8 and caspase-3, but not caspase-9. Unlike hypoxia, low glucose promoted caspase-9 activation during TRAIL treatment. These results suggest that hypoxia or low glucose-augmented TRAIL cytotoxicity is mediated through the mitochondria-independent pathway or -dependent pathway, respectively. (Mol Cell Biochem **270**: 89–97, 2005)

Introduction

For 40 years it has been noted that the interstitial fluid of a solid tumor is often characterized by a very low concentration of free glucose [1], a high concentration of lactic acid [2, 3], low-oxygen tensions [4], and low-extracellular pH [5]. This is due to severe architectural and functional abnormalities of the capillary network of most tumors. Tumor venules are tortuous, elongated, and often dilated. The host vessels per unit tumor mass do not increase in number, thus leading to a reduction of the available exchange area for oxygen, nutrients, hormones, growth factors, and waste products [6]. These characteristic features can markedly affect the therapeutic response [7, 8]. It is, therefore, very important to understand the role of tumor microenvironment in the biochemical functions of anticancer agents in tumors to improve their efficacy in cancer therapy. Recently, we demonstrated that low glucose as well as low-extracellular pH augment the effect of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a potent anticancer agent, to induce apoptosis [9, 10]. In this

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study, we investigated whether hypoxia augments TRAIL-induced cytotoxicity. We also compared the effect of hypoxic on TRAIL cytotoxicity to that of low glucose in order to assess the differential effectiveness of this anticancer agent in tumor microenvironment.

TRAIL/APO-2L is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family. TRAIL is a 281-amino acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand (FasL) and TNF, the C-terminal extracellular region of TRAIL (amino acids 114–281) exhibits a homotrimeric subunit structure [11]. However, unlike FasL and TNF, it induces apoptosis in a variety of tumor cell lines more efficiently than normal cells [12]. The apoptotic signal induced by TRAIL is transduced by its binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling [13–19]. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif [16]. Differential sensitivity between normal and tumor cells to TRAIL has been explained by the presence of large numbers of the decoy receptors on normal cells [20, 21]. Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines [22] and melanoma cell lines [23]. This discrepancy indicates that other factors such as death inhibitors including FLICE-inhibitory protein (FLIP) [23], Fas-associated protein (FAP-1) [24], Bcl-2 [25], Bcl-X_L [25], Bruton's tyrosine kinase (BTK) [26], silencer of death domain (SODD) [27], toso [28], inhibitor of apoptosis (IAP) [29], X-linked inhibitor of apoptosis (XIAP) [30], and survivin [31] are also involved in the differential sensitivity to TRAIL. Previous studies show that chemotherapeutic agents [22, 23, 32] and ionizing radiation [33] can sensitize TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP [23] or increasing DR5 gene expression in response to genotoxic stress [32–34]. In this study, we observed that low glucose, but not hypoxia, augments TRAIL-induced apoptotic death by promoting the mitochondria-mediated caspase signal transduction pathway.

Materials and methods

Cell culture and survival assay

Human prostate adenocarcinoma DU-145 cells were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD, U.S.A.) with 10% fetal bovine serum (HyClone, Logan, Utah, U.S.A.) and 26 mM sodium bicarbonate for monolayer cell culture. Human prostate adenocarcinoma LNCaP cells were cultured in RPMI-1640 medium (Gibco BRL) with 10% fetal bovine serum (HyClone), 1 mM L-glutamine and 26 mM sodium bicarbonate for monolayer cell culture. The dishes containing cells were kept in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2. Two days prior to the experiment, cells were plated into 60-mm dishes. For Trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 mL of medium, 0.5 mL of 0.4% Trypan blue solution and 0.3 mL of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. At least 300 cells were counted for survival determination.

Glucose deprivation

Cells were rinsed three times with PBS and then exposed to glucose-free DMEM containing 10% dialyzed fetal bovine serum (Gibco BRL).

Hypoxic treatment

For treatment of hypoxia, cells were plated in a glass T-flask instead of a plastic flask due to trace amounts of oxygen in the plastic. Following an overnight incubation, the glass flask was sealed with a rubber plug and flushed with a gas mixture of either 1 or 0.001% oxygen balanced with 5% CO_2 and nitrogen (Puritan Medical Products, Linthicum Height, MD). A selected gas mixture was flushed through the inlet and outlet needles for 1 h with constant shaking, while the control group was exposed to the air (21% oxygen). Our previous experiments confirmed that radiobiological hypoxia is achieved by flushing a nitrogen gas (less than 10 ppm oxygen) for 45 min. At the end of 1 h gas flushing, TRAIL was added through the outlet needle to make the final concentration of 50–200 ng/mL, and the gas flushing continued for another 1 h after the TRAIL addition. Then, the outlet and inlet needles were removed to maintain the same gas condition in the flask.

Production of recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into a pET-23d (Novagen, Madison, WI, U.S.A.) plasmid, and His-tagged TRAIL protein was purified using the Qiaexpress protein purification system (Qiagen, Valencia, CA, U.S.A.) [35].

Antibodies

Polyclonal anti-caspase-3 and anti-Bcl-X_L antibodies were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.).

Anti-Akt and anti-phospho-Akt from Cell Signaling (Beverly, MA, U.S.A.), anti-cIAP-1 and anti-cIAP-2 from R&D Systems (Minneapolis, MN, U.S.A.), and anti-FLIP from Calbiochem (Darmstadt, Germany) were used. Monoclonal antibodies were purchased from the following companies: anti-caspase-9 and anti-caspase-8 from Upstate Biotechnology (Lake Placid, NY, U.S.A.), anti-cytochrome c from PharMingen (San Diego, CA, U.S.A.), anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.), and anti-actin and anti-Bcl-2 from ICN (Costa Mesa, CA, U.S.A.).

Protein extracts and polyacrylamide gel electrophoresis (PAGE)

Cells were lysed with 1 × Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and an equal amount of protein was loaded on 8–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli using a Hoefer gel apparatus.

Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 7.5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 1 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, U.S.A.).

Results

Effect of low glucose on TRAIL-induced apoptotic death

Our recent studies have demonstrated that TRAIL-induced cytotoxicity as well as caspase activation is promoted by lowering the concentration of glucose [9]. We further extended our studies to examine whether the effects observed would occur as a function of glucose concentration. As DU-145 cells were exposed to medium containing decreasing concentrations of glucose, they exhibited a dose-responsive in-
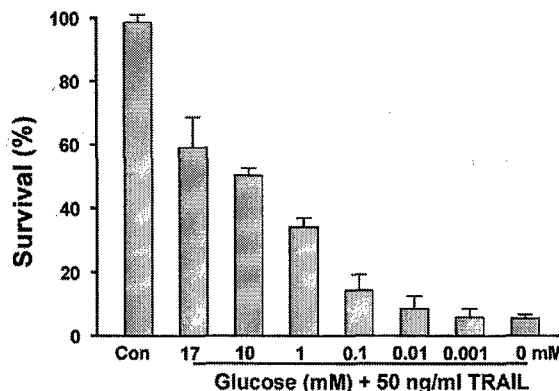
Fig. 1. Effect of various concentrations of glucose on TRAIL-induced cytotoxicity in DU-145 cells. Cells were treated with TRAIL (50 ng/mL) in the presence of various concentrations of glucose (0–17 mM) for 4 h. Osmolarity of the medium was adjusted by adding NaCl. Cell survival was analyzed by Trypan blue exclusion assay. Error bars represent ±S.D.

crease in sensitivity to killing by TRAIL (Fig. 1). Additional studies were designed to determine whether the combination of TRAIL and glucose deprivation enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis (Fig. 2). Glucose deprivation alone failed to induce PARP cleavage (data not shown). In contrast, TRAIL (50 ng/mL) in complete medium (17 mM glucose) caused PARP cleavage (lane 2 in Fig. 2A). PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL. PARP cleavage was markedly enhanced when TRAIL and low glucose were combined; cleavage increased as the glucose concentration decreased (Fig. 2A). These results show that low glucose significantly enhances TRAIL-induced apoptosis. We further examined the effect of low glucose on TRAIL-induced activation of caspases. DU-145 cells were treated with 50 ng/mL TRAIL in the presence of various concentrations of glucose (0–17 mM) for 4 h (Fig. 2B). Procaspase-8 (55 kDa) was cleaved to the intermediate (43 and 41 kDa) and active forms (18 kDa) by treatment with TRAIL (lane 2 in Fig. 2B). The combined treatment with TRAIL and low glucose resulted in an increase in caspase-8 activation compared to TRAIL alone (Fig. 2B). Similar results were observed in the activation of caspase-3 and caspase-9. Low glucose promoted the TRAIL-induced activation of caspase-3 and caspase-9. The activation was increased as the glucose concentration decreased (Fig. 2B).

Effect of hypoxia on TRAIL-induced apoptotic death

The effect of hypoxia on cell death in conjunction with TRAIL was assessed by colony formation in DU-145 cells

92

A

Glucose (mM) + 50 ng/ml TRAIL

Con 17 10 1 0.1 0.01 0.001 0 mM

PARP ← 116 kDa
← 85 kDa

Actin ← 42 kDa

1 2 3 4 5 6 7 8

B

Glucose (mM) + 50 ng/ml TRAIL

Con 17 10 1 0.1 0.01 0.001 0

Caspase -8 ← 55 kDa
← 43 /41 kDa
← 18 kDa

Caspase -3 ← 32 kDa
← 17 kDa

Caspase -9 ← 48 kDa
← 37 kDa

Actin ← 42 kDa

1 2 3 4 5 6 7 8

Fig. 2. Effect of various concentrations of glucose on TRAIL-induced proteolytic cleavage of PARP (A) and activation of caspases (B). DU-145 cells were treated with 50 ng/mL TRAIL for 4 h in the presence of various concentrations of glucose (0–17 mM) and then harvested. Lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. Panel A, The upper band indicates 116 kDa PARP whereas the lower band indicates the 85 kDa apoptosis-related cleavage fragment. Panel B, Antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (43 and 41 kDa), and active subunit (18 kDa). Anti-caspase-3 antibody detects both inactive form (32 kDa) and active form (17 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and intermediate form (37 kDa). Con, untreated control cells. Actin was used to confirm that similar amounts of protein were loaded in each lane.

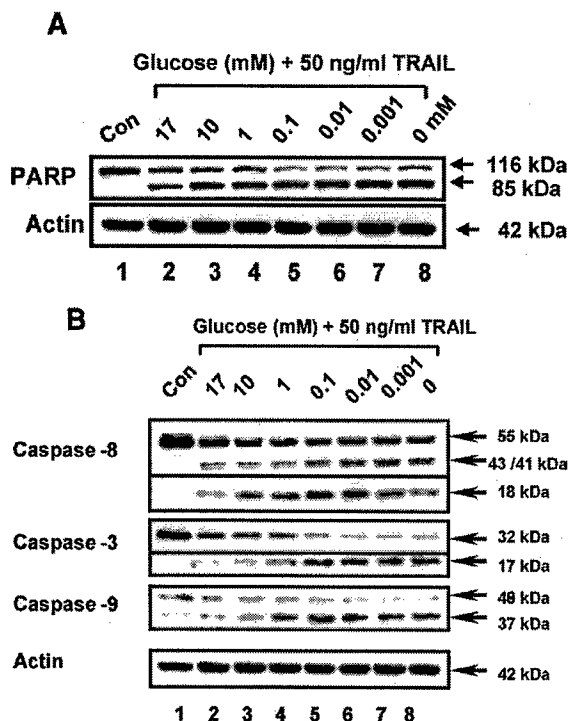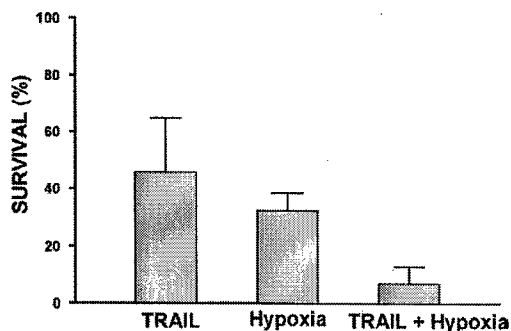*Fig. 3.* Effect of hypoxia on TRAIL-induced cytotoxicity in DU-145 cells. Cells were treated with TRAIL (50 ng/mL) alone, hypoxia (0.001% O_2) alone, or TRAIL in combiantion with hypoxia for 4 h. Survival was determined by colony formation assay. Survival was normalized for plating efficiency. Error bars represent ±SD.

(Fig. 3). When cells were treated with 50 ng/mL TRAIL or hypoxia (0.001% oxygen) for 4 h, the survival was 45.7% or 32.5%, respectively. The combination of TRAIL and hypoxia synergistically increased cell death compared with cells treated with TRAIL alone or hypoxia alone. These results indicate that TRAIL cytotoxicity was significantly enhanced by the combined treatment. We further examined the effect of hypoxia on TRAIL-induced PARP cleavage and caspase activation. Figure 4A shows that hypoxia alone failed to induce PARP cleavage and caspase activation. However, hypoxia enhanced TRAIL-induced PARP cleavage as well as the activation of caspase-8 and caspase-3 in DU-145 cells (lane 2 vs. lane 4 in Fig. 4A). In contrast, anoxia failed to promote caspase-9 activation (lane 4 in Fig. 4B). Unlike hypoxia, glucose deprivation promoted caspase-9 activation during TRAIL treatment (lane 5 in Fig. 4B). To examine whether our findings can be generalized for more than one cell line, human prostate adenocarcinoma LNCaP cells were employed. Figure 4C also shows that, like in DU-145 cells, hypoxia enhanced TRAIL-induced PARP cleavage in LNCaP cells. However, recent studies have shown that hypoxia protects cells from TRAIL-induced apoptotic death [36]. To investigate whether this discrepancy is due to different oxygen tension or different oxygen exposure time, we used 1% oxygen or 24 h pre-exposure of hypoxia. Figure 5 shows that an oxygen concentration of 1% still promoted TRAIL-induced apoptosis when DU-145 cells were exposed to 1% oxygen with or without TRAIL (50 or 200 ng/mL) for 4 h. Similar results were observed when cells were exposed to 1% oxygen for 24 h followed by treatment with TRAIL (50 ng/mL) in hypoxia (Fig. 6A). Similar results were also consistently observed in various concentrations of TRAIL (50–200 ng/mL) (Fig. 6B).

Effect of low glucose in combination with hypoxia on TRAIL-induced apoptosis

The long-term goal of our studies is to understand how tumor physiology can impact TRAIL-induced cytotoxicity. Conditions that cause tumor cells to experience low glucose would also result in low oxygen tensions. We examined whether low oxygen tensions alter glucose deprivation-promoted TRAIL cytotoxicity. The first step in this study was to examine whether hypoxia in combination with glucose deprivation cause caspase activation and PARP cleavage. Figure 7A shows that hypoxia alone, glucose deprivation alone, or combined hypoxia and low glucose (0.01–10 mM) did not cause PARP cleavage and activation of caspase-8 and caspase-3. In contrast, PARP cleavage and activation of caspase-8 and caspase-3 occurred by treatment with TRAIL in the absence of glucose (lane 8 in Fig. 7A). The next step was to investigate whether hypoxia
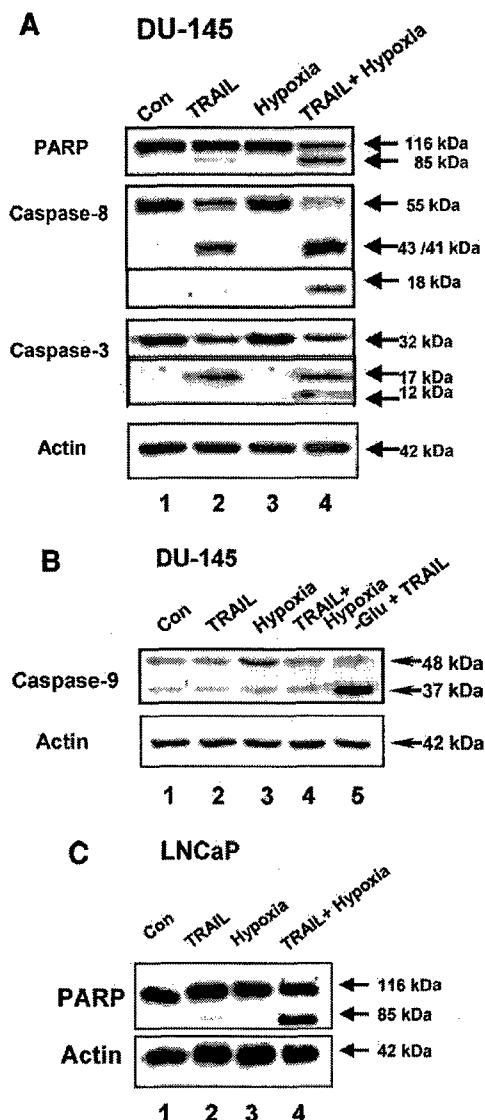
A DU-145

PARP ← 116 kDa / 85 kDa

Caspase-8 ← 55 kDa / ←43/41 kDa / ← 18 kDa

Caspase-3 ←32 kDa / ←17 kDa / 12 kDa

Actin ←42 kDa

1 2 3 4

B DU-145

Caspase-9 ←48 kDa / ←37 kDa

Actin ←42 kDa

1 2 3 4 5

C LNCaP

PARP ← 116 kDa / ← 85 kDa

Actin ← 42 kDa

1 2 3 4

Fig. 4. Effect of hypoxia (0.001% O_2) on TRAIL-induced proteolytic cleavage of PARP and activation of caspase-8, -3, and -9. Panels A and B, DU-145 cells were treated with TRAIL (50 ng/mL) alone, hypoxia alone, or TRAIL + hypoxia for 4 h. Panel C, LNCaP cells were treated with TRAIL (50 ng/mL) alone, hypoxia alone, or TRAIL + hypoxia for 4 h. Lysates containing equal amounts of protein (20 μg/mL) were separated by SDS-PAGE and immunoblotted. PARP, caspase-8, caspase-3, and caspase-9 were detected as described in Fig. 2. Con, untreated control cells. —Glu + TRAIL, cells were treated with 50 ng/mL TRAIL in the absence of glucose. Actin is shown as an internal standard.

alters the combined treatment with TRAIL and low glucose-induced PARP cleavage and caspase activation as previously shown in Fig. 2. Figure 7B shows that hypoxia in combination with low glucose enhanced TRAIL-induced

PARP cleavage and activation of caspase-8, caspase-9 and caspase-3.

Our previous studies show that low glucose-enhanced TRAIL cytotoxicity and caspase activation are mediated through the Akt-FLIP pathway [9]. Glucose deprivation in combination with TRAIL may disrupt the PI(3)K pathway and subsequently reduce Akt phosphorylation (causing inactivation of Akt) and consequently downregulate FLIP gene expression. To test this possibility, DU-145 cells were treated with 50 ng/mL TRAIL in the presence of various concentrations of glucose (0–17 mM) for 4 h. Total cell lysates were harvested and immunoblotted. Western blot analysis showed that low glucose in combination with TRAIL effectively dephosphorylated Akt at serine 473 (S473) and reduced the intracellular level of FLIP, but not that of Akt (Fig. 8A). The dephosphorylation of Akt and reduction of FLIP level were increased as the glucose concentration decreased. Figure 8A shows that low glucose in combination with TRAIL dephosphorylated Akt and reduced the level of $FLIP_L$. We examined whether hypoxia-augmented TRAIL cytotoxicity is also mediated through the Akt-FLIP pathway. Figure 8B shows that hypoxia alone or combined hypoxia and TRAIL treatment did not dephosphorylate Akt at serine 473 (lanes 3 and 4 in Fig. 8B). They did not alter the level of FLIP, either (lanes 3 and 4 in Fig. 8B). These results clearly demonstrate that hypoxia-enhanced TRAIL cytotoxicity is not mediated through the Akt-FLIP pathway. Previous studies reveal that the inhibition of the Akt pathway suppresses the activation of NF-κB and subsequently inhibits the expression of anti-apoptotic family proteins (IAPs, Bcl-X_L, and FLIP) [37]. We further extended our studies whether low glucose/hypoxia-promoted TRAIL cytotoxicity is mediated through altering the expression of anti-apoptotic proteins. Figure 8C shows that low glucose and hypoxia did not significantly alter the levels of IAP-1, IAP-2, Bcl-2, and Bcl-X_L. Interestingly, TRAIL in combination with hypoxia and low glucose significantly reduced the level of IAP-1.

Discussions

We previously observed that TRAIL-induced caspase-3 activation and apoptosis are mediated through two different apoptotic pathways, mitochondria-dependent and mitochondria-independent [35]. Our studies clearly demonstrate that low glucose-augmented TRAIL cytotoxicity is mediated through promoting caspase-9 activation, part of the mitochondria-dependent pathway. However, unlike low glucose, hypoxia-promoted TRAIL cytotoxicity is not associated with caspase-9 activation. These results suggest that low glucose and hypoxia enhance TRAIL-induced apoptotic death by promoting the mitochondria-dependent pathway and the mitochondria-independent pathway, respectively.
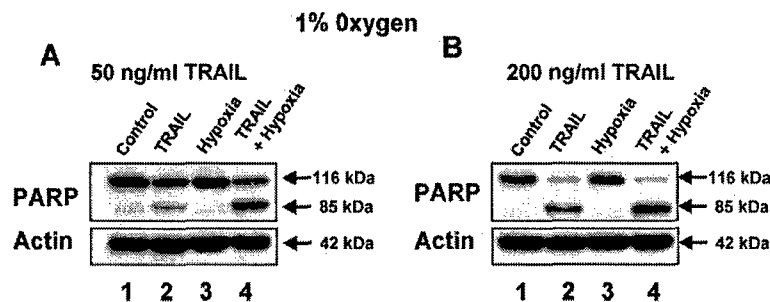
1% Oxygen

A 50 ng/ml TRAIL

Control, TRAIL, Hypoxia, TRAIL + Hypoxia

PARP ← 116 kDa
← 85 kDa

Actin ← 42 kDa

1 2 3 4

B 200 ng/ml TRAIL

Control, TRAIL, Hypoxia, TRAIL + Hypoxia

PARP ← 116 kDa
← 85 kDa

Actin ← 42 kDa

1 2 3 4

Fig. 5. Effect of hypoxia (1% O$_2$) on TRAIL-induced proteolytic cleavage of PARP in DU-145 cells. Cells were treated with TRAIL (Panel A, 50 ng/mL; Panel B, 200 ng/mL) alone, hypoxia alone, or TRAIL + hypoxia for 4 h. Lysates containing equal amounts of protein (20 μg/mL) were separated by SDS-PAGE and immunoblotted with anti-PARP or anti-actin antibody. Actin is shown as an internal standard.

A

Control, TRAIL, Hypoxia (4 h), Hypoxia (4 h) + TRAIL, Hypoxia (28 h), Hypoxia (24 h) → TRAIL + Hypoxia (4 h)

PARP

Actin

1 2 3 4 5 6

B

Hypoxia (24 h) → TRAIL+Hypoxia

TRAIL (ng/ml)   -   50 100 150 200

PARP ← 116 kDa
← 85 kDa
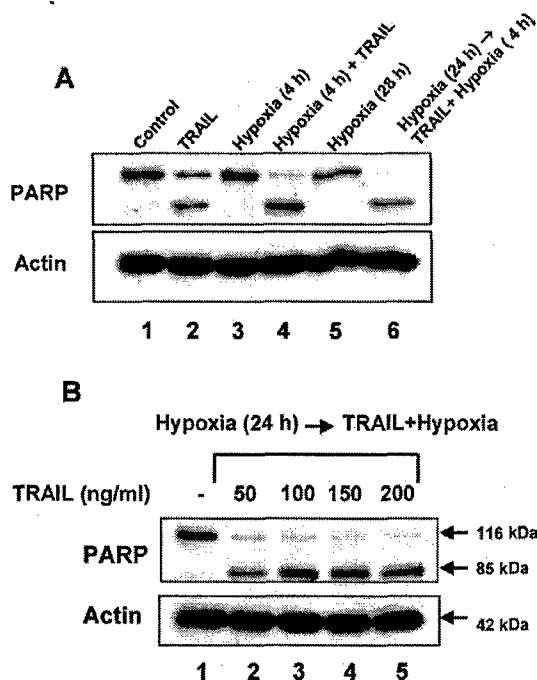
Actin ← 42 kDa

1 2 3 4 5

Fig. 6. Effect of pre-exposure to hypoxia (1% O$_2$) on TRAIL-induced proteolytic cleavage of PARP in DU-145 cells. Panel A, Cells were treated with TRAIL (50 ng/mL) alone, hypoxia for 4 h, TRAIL + hypoxia for 4 h, hypoxia for 28 h, or hypoxia for 24 h prior to treatment with TRAIL for 4 h. Panel B, Cells were exposed to hypoxia for 24 h prior to various concentrations of TRAIL (50–200 ng/mL). Lysates containing equal amounts of protein (20 μg/mL) were separated by SDS-PAGE and immunoblotted with anti-PARP or anti-actin antibody. Actin is shown as an internal standard.

Several researchers reported that the Fas-associated death domain (FADD) is required for TRAIL-induced apoptosis [38]. TRAIL triggers apoptosis by recruiting the apoptosis initiator procaspase-8 through the adaptor FADD [39].

A

Hypoxia + Glucose (mM)

Con, Hypoxia, -Glu, 10, 1, 0.1, 0.01, -Glu + TRAIL

PARP ← 116 kDa
← 85 kDa

Caspase-8 ← 55 kDa
← 43/41 kDa

Caspase-3 ← 32 kDa

Actin ← 42 kDa

1 2 3 4 5 6 7 8

B

Con, TRAIL, Hypoxia, -Glu, Hypoxia + TRAIL, -Glu + TRAIL, -Glu + Hypoxia + TRAIL

PARP ← 116 kDa
← 85 kDa

Caspase-8 ← 54/55 kDa
← 41/43 kDa

Caspase-9 ← 48 kDa
← 37 kDa

Caspase-3 ← 32 kDa

← 17 kDa
← 12 kDa

Actin ← 42 kDa

1 2 3 4 5 6 7
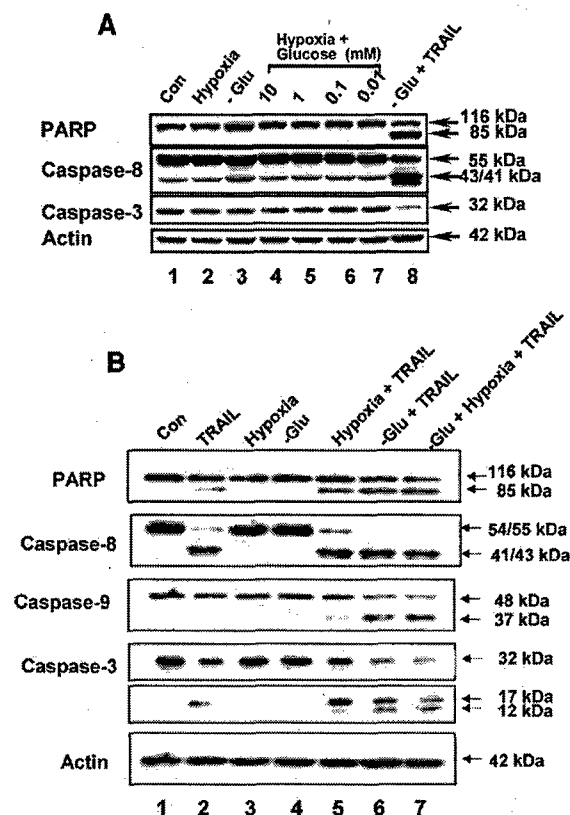
Fig. 7. Various combinational treatments-induced PARP cleavage and caspase activation in DU-145 cells. Panel A, Cells were exposed to hypoxia, glucose derpivation, hypoxia + various concentrations of glucose, or TRAIL (50 ng/mL) + glucose deprivation for 4 h. Panel B, Cells were exposed to TRAIL (50 ng/mL), hypoxia, low glucose, hypoxia + TRAIL (50 ng/mL), low glucose + TRAIL (50 ng/mL), or TRAIL (50 ng/mL) + hypoxia + low glucose for 4 h. Lysates containing equal amounts of protein (20 μg/mL) were separated by SDS-PAGE and immunoblotted. PARP, caspase-8, caspase-3, and caspase-9 were detected as described in Fig. 2. Con, untreated control cells. Actin is shown as an internal standard.

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Fig. 8. Effect of various combination treatments on anti-apoptotic proteins. DU-145 cells were treated with lowglucose + TRAIL (50 ng/mL), TRAIL (50 ng/mL), hypoxia, hypoxia + TRAIL for 4 h (Panels A and B) or various combinat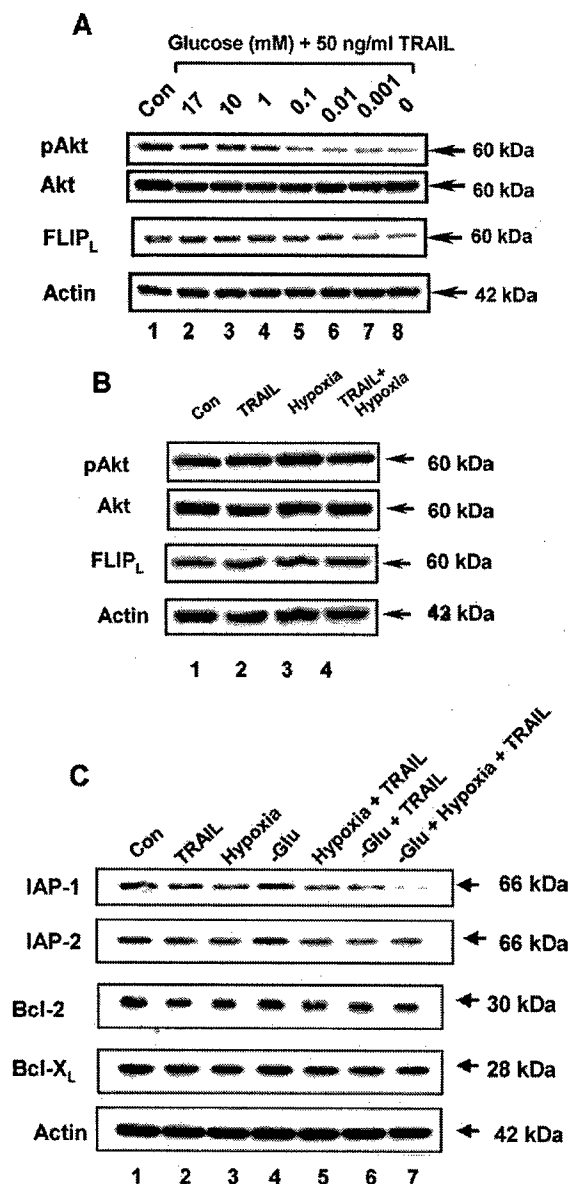ions for 3 h (Panel C). Lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, anti-FLIP$_L$, anti-IAP-1, anti-IAP-2, anti-Bcl-2, or anti-Bcl-X$_L$ antibody. Con, untreated control cells. Actin was shown as an internal standard.

Caspase-8 can directly activate downstream effector caspases including procaspase-3, procaspase-6, and procaspase-7 [40]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome c release [41].

Cytochrome c in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [42]. The activation of caspases is counteracted by anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-X$_L$). The Bcl-2 family proteins heterodimerize with pro-apoptotic members of the family (Bax, Bak) and interfere with release of cytochrome c by pore-forming proteins (Bid, Bik) [43]. Members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP) can directly bind and inhibit activation of caspases including caspase-3, caspase-7 and caspase-9 [44]. The expression of these Bcl-2 family and IAP family proteins, both of which inhibit apoptosis, is promoted by NF-κB, a family of dimeric transcription factors [37]. Several studies have shown that the NF-κB signal is regulated by Akt. Akt is activated by phosphoinositide-dependent kinase-1 (PDK1) through phosphorylation at threonine 308 and serine 473 [45]. Recent studies demonstrated that Akt activity is also regulated by Ser/Thr phosphatase, PP1 [46] or PP2A [47, 48]. A number of proapoptotic proteins have been identified as direct Akt substrates, including BAD, caspase-9, GSK-3 and Forkhead transcription factors [49–55]. The proapoptotic function of these molecules is suppressed on phosphorylation by Akt. Recent studies also show that Akt induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB [56], which, as was stated above, promotes Bcl-2 family and IAP family proteins, both of which inhibit apoptosis. In this study, we clearly demonstrate that low glucose in combination with TRAIL reduces the level of phosphorylated Akt as well as FLIP$_L$ protein. We previously suggested that glucose deprivation-enhanced TRAIL cytotoxicity is mediated through the ceramide-Akt-FLIP pathway [9].

Recent studies have revealed that hypoxia protects tumor cells from TRAIL-induced apoptosis as well as elevates the expression of antiapoptotic proteins such as Bcl-2, Bcl-X$_L$, and IAP family members in human lung carcinoma A549 cells [36]. However, our data clearly reveal that hypoxia promotes TRAIL-induced apoptotic death in human prostate adenocarcinoma DU-145 as well as LNCaP cells (Figs. 3–6). We also observed that there was no difference between short-term exposure (4 h) and long-term exposure (24 h) to hypoxia prior to TRAIL treatment (Fig. 6). In addition, we were not able to find a significant increase in the levels of these anti-apoptotic proteins during hypoxia or in combination with low glucose (Fig. 8C). Similar results are observed in a variety of cell lines under various oxygen tensions [57]. At the present time, we can only speculate that this discrepant effect of hypoxia on TRAIL-induced apoptotic death is due to different cell lines. The discrepancy needs to be further studied and the models proposed here provide a framework for future studies.

Acknowledgments

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Role of HER-2/neu Signaling in Sensitivity to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand: Enhancement of TRAIL-Mediated Apoptosis by Amiloride

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Abstract   Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in numerous transformed cell lines but not in most normal cells. Although this selectivity offers a potential therapeutic application in cancer, not all cancers are sensitive to TRAIL-mediated apoptosis. In this study, we observed that amiloride, a current clinically used diuretic drug, which had little or no cytotoxicity, sensitized TRAIL-resistant human prostate adenocarcinoma LNCaP and human ovarian adenocarcinoma SK-OV-3 cells. The TRAIL-mediated activation of caspase, and PARP cleavage, were promoted in the presence of amiloride. Western blot analysis showed that combined treatment with TRAIL and amiloride did not change the levels of TRAIL receptors (DR4, DR5, and DcR2) and anti-apoptotic proteins (FLIP, IAP, and Bcl-2). However, amiloride dephosphorylated HER-2/neu tyrosine kinase as well as Akt, an anti-apoptotic protein. Interestingly, amiloride also dephosphorylated PI3K and PDK-1 kinases along with PP1α phosphatase. In vitro kinase assay revealed that amiloride inhibited phosphorylation of kinase as well as phosphatase by competing with ATP. Taken together, the present studies suggest that amiloride enhances TRAIL-induced cytotoxicity by inhibiting phosphorylation of the HER-2/neu-PI3K-Akt pathway-associated kinases and phosphatase. J. Cell. Biochem. 9999: 1–14, 2005.   © 2005 Wiley-Liss, Inc.

Key words: amiloride; TRAIL; apoptosis; caspase; Akt; PDK-1; HER-2/neu

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Amiloride (3,5 diamino-6-chloro-N-(diamino-methylene) pyrazinecarboximide) is a diuretic drug and has been known to produce a low pH in the intracellular environment by blocking the intracellular pH regulatory mechanism, the Na^+/H^+ antiport, on the plasma membrane surfaces of mammalian cells [Zhuang et al., 1984; Mahnensmith and Aronson, 1985]. Amiloride is also known to inhibit tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], Na^+/Ca^{2+} exchange [Smith et al., 1982], (Na^+-K^+)-ATPase [Soltoff and Mandel, 1983], and serine kinase activity [Ralph et al., 1982]. Previous studies demonstrated that amiloride enhances thermal killing and inhibits development of thermotolerance [Miyakoshi et al., 1986; Kim et al., 1991]. Recently, we observed that amiloride enhances TRAIL-induced apoptotic death in human prostate adenocarcinoma DU-145 cells (an androgen-independent cell line expressing low levels of HER-2/neu) [Kim and Lee, 2005]. In this study, we examined whether amiloride can also sensitize TRAIL-resistant human prostate

adenocarcinoma LNCaP cells (an androgen-dependent cell line expressing high levels of HER-2/neu) and human ovarian adenocarcinoma SK-OV-3 cells (a cell line expressing high levels of HER-2/neu).

HER-2/neu (also known as erbB2) is a gene in the epidermal growth factor receptor (EGFR) family. The HER-2/neu gene encodes a 185 kDa transmembrane receptor tyrosine kinase. Unlike the other epidermal growth factor receptors (EGFR), HER-2/neu has an intrinsic tyrosine kinase activity that activates PI(3)K in the absence of ligand [Fruman et al., 1998] PI(3)K consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (P110) [Rodriguez-Viciana et al., 1996]. PI(3)K phosphorylates phosphoinositides at the 3'-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (PIP$_3$) [Rameh and Cantley, 1999]. PIP$_3$ facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology (PH) domain of Akt [Rameh and Cantley, 1999]. Akt is activated by phosphoinositide-dependent kinase-1 (PDK-1) through phosphorylation at threonine 308 and serine 473 [Alessi et al., 1997]. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspsase-9, and Forkhead transcription factors [Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998; Brunet et al., 1999; Hetman et al., 2000]. The pro-apoptotic function of these molecules is suppressed upon phosphorylation by Akt. Recent studies also show that Akt induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB [Ozes et al., 1999]. Nam et al. [2002] and Panka et al. [2001] reported that the PI(3)K-Akt-NF-κB pathway may regulate the expression of FLICE-inhibitory protein (FLIP), an anti-apoptotic molecule. Several studies reveal that TRAIL induces apoptosis in a wide variety of tumor cells, but does not cause toxicity to most normal cells. However, recent studies reveal that a polyhistidine-tagged TRAIL induces apoptosis in normal human hepatocytes in culture [Jo et al., 2000]. This is probably due to an aberrant conformation and subunit structure of TRAIL in the presence of low zinc concentrations [Lawrence et al., 2001]. In contrast, native-sequence, non-tagged recombinant TRAIL, when produced under optimized zinc concentrations, is markedly more active against tumor cells than the polyhistidine-tagged ligand, but has minimal toxicity toward human hepatocytes in vitro [Lawrence et al., 2001]. Moreover, preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but no cytotoxicity to normal organs or tissue [Walczak et al., 1999]. In addition, unlike tumor necrosis factor (TNF) and Fas ligand (FasL), TRAIL mRNA is expressed constitutively in many tissues [Wiley et al., 1995; Pitti et al., 1996].

Recent studies also revealed that TRAIL, which is constitutively expressed on murine natural killer cells in the liver, plays an important role in surveillance of tumor metastasis [Takeda et al., 2001]. The apoptotic signal of TRAIL is transduced by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling [Degli-Esposti et al., 1997a,b; Marsters et al., 1997; Pan et al., 1997a,b; Sheridan et al., 1997; Walczak et al., 1997]. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif [Pan et al., 1997b]. Differential sensitivity between normal and tumor cells to TRAIL has been explained by the presence of a high concentration of the decoy receptors in normal cells [Gura, 1997; Ashkenazi and Dixit, 1999]. Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines [Keane et al., 1999]. This discrepancy indicates that other factors such as death inhibitors (FLIP, FAP-1, or IAP) are also involved in the differential sensitivity of TRAIL. Previous studies demonstrated that the Fas-associated death domain (FADD) is also required for TRAIL-induced apoptosis [Kischkel et al., 2000]. TRAIL triggers apoptosis by recruiting the apoptosis initiator procaspase-8 through the adaptor FADD

[Bodmer et al., 2000]. Caspase-8 can directly activate downstream effector caspases including procaspase-3, -6, and -7 [Cohen, 1997]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome c release [Li et al., 1997]. Cytochrome c in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [Slee et al., 1999].

The activation of caspases is counteracted by anti-apoptotic molecules of the Bcl-2 and IAP families [Tamm et al., 2000]. The Bcl-2 family proteins (Bcl-2, Bcl-X_L) heterodimerize with pro-apoptotic members of the family (Bax, Bak) and interfere with release of cytochrome c by pore-forming proteins (Bid, Bik) [Fruman et al., 1998]. Members of the inhibitor of apoptosis (IAP) family protein (c-IAP1, c-IAP2, XIAP) can directly bind and inhibit activation of caspases including caspase-3, -7 and -9 [Roy et al., 2001]. The expression of these Bcl-2 family and IAP family anti-apoptotic proteins is promoted by NF-κB, a family of dimeric transcription factors [Chen et al., 2000]. The NF-κB family of proteins, including NF-κB1, NF-κB2, RelA, RelB, and c-Rel, can form homo- and heterodimers in vitro, except for RelB. In mammals, the most widely distributed NF-κB is a heterodimer composed of p50 and p65 (also called RelA) subunits [Baeuerle and Baltimore, 1989]. NF-κB activity is regulated by the IκB family of proteins that interacts with and sequesters the transcription factor in the cytoplasm. IκB proteins become phosphorylated by the multisubunit IκB kinase (IKK) complex, which subsequently targets IκB for ubiquitination and degradation by the 26S proteasome [Zandi and Karin, 1999].

In this study, we postulate that blockage of HER-2/neu-mediated survival signals can sensitize HER-2/neu overexpressing cancers to TRAIL-induced cell death. Our data revealed that amiloride, which is known to inhibit tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], inhibits the HER-2/neu associated PI(3)K-Akt signal transduction pathway and thus renders the cell more sensitive to TRAIL-induced apoptotic death.

MATERIALS AND METHODS

Cell Culture and Survival Assay

Human prostate adenocarcinoma LNCaP cell line and two human ovarian adenocarcinoma cell lines, OVCAR-3 and SK-OV-3, were obtained from the American Tissue Type Culture Collection (Manassas, VA). LNCaP and SK-OV-3 cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (HyClone, Logan, Utah), 1 mM L-glutamine, and 26 mM sodium bicarbonate for monolayer cell culture. OVCAR-3 cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum, 10 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO), 1 mM sodium pyruvate (Gibco BRL), 25 mM glucose, 17.9 mM sodium bicarbonate, and 0.01 mg/ml human recombinant insulin (Sigma). The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. One day prior to the experiment, cells were plated into 60-mm dishes. For trypan blue exclusion assay [Burow et al., 1998], trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. At least 300 cells were counted for each survival determination.

Drug Treatment

Wortmanin, sodium orthovanadate, 3-amino-6-chloro-5-(1-homopiperidyl)-N-(diaminomethylene) pyraxinecarboxamide (HMA), and 3,5-diamino-6-chloro-N-(diaminomethylene) pyrazinecarboximide (amiloride) were obtained from Sigma Chemical Co. LY294002 was purchased from Cell Signaling (Beverly, MA), and okadaic acid from Calbiochem (San Diego, CA). A stock solution was prepared in dimethylsulfoxide (DMSO).

Production of Recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114−281) obtained by RT-PCR was cloned into pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the Qiaexpress protein purification system (Qiagen, Valencia, CA).

TUNEL Assay

For detection of apoptosis by the TUNEL method, cells were plated in slide chambers. After treatment, cells were fixed with 70% ethanol in PBS. Cells were washed once, permeabilized by incubating with 100 μl of 0.1%

Triton X-100, 0.1% sodium citrate, and then washed twice in PBS. The TUNEL reaction was carried out at 37°C for 1 h with 0.3 nmol of fluorescein isothiocyante-12-dUTP, 3 nmol of dATP, 2 μl pf $CoCl_2$, 25 U of terminal deoxynucleotidyl transferase, and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) in a total reaction volume of 50 μl. The reaction was stopped with 2 μl of 0.5M EDTA. Cells were observed under a fluorescence microscope.

Morphological Evaluation

Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome 4′,6-diamidino-2-phenylindole (DAPI). Cells were grown on glass coverslips at a density of 2×10^5 cells/well. Cells treated with TRAIL, amiloride, or TRAIL in combination with amiloride for 4 h. Cells were washed twice with PBS and fixed by incubation in 70% ethanol for 30 min. Following washes with PBS, cells were incubated in 1 μg/ml DAPI solution for 30 min in the dark. Coverslips were then washed with PBS and analyzed by fluorescence microscopy.

UV Irradiation

For UV irradiation experiments, cells were exposed to UV-C at an intensity of 100 J/m^2 in a UV cross-linker (Spectronics Corporation, Westbury, New York).

Antibodies

Polyclonal anti-phospho(Tyr508)-PI3K, anti-Bcl-XL, anti-Bad, anti-caspase-3, and anti-caspase-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA), anti-DR5 and anti-DcR2 from StressGen (Victoria, BC, Canada), anti-DR4 from Upstate Biotechnology (Lake Placid, NY), anti-HER-2/neu from Dako-Cytomation (Carpinteria, CA), anti-cIAP-1 and anti-cIAP-2 from R&D Systems (Minneapolis, MN), anti-phospho(Tyr1248)-HER-2/neu, anti-phospho(Ser473)-Akt, anti-Akt, anti-phospho-(Ser241)-PDK-1, anti-PDK-1, anti-phospho-(Thr320)-PP1, and anti-PP1 from Cell Signaling, and anti-FLIP from Calbiochem. Monoclonal antibodies were purchased from the following companies: anti-PI3K from Santa Cruz, anti-caspase-8 from Upstate Biotechnology, anti-cytochrome c from PharMingen (San Diego, CA), anti-PARP from Biomol Research Laboratory (Plymouth Meeting, PA), and anti-Bcl-2 and anti-actin from ICN (Costa Mesa, CA).

Protein Extracts and Polyacrylamide Gel Electrophoresis (PAGE)

Cells were lysed with 1× Laemmli lysis buffer (2.4M glycerol, 0.14M Tris, pH 6.8, 0.21M sodium dodecyl sulfate, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer containing 1.28M β-mercaptoethanol, and equal amounts of protein were loaded on 8%–12% sodium dodecyl sulfate (SDS)–polyacrylamide gels. SDS–PAGE analysis was performed according to Laemmli [1970] using a Hoefer gel apparatus.

Immunoblot Analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL).

In Vitro Enzyme Assays

For in vitro PP1 phosphatase assays, 10 ml of active PP1a proteins (1 U/ml; Upstate Biotechnology) were incubated with 3 ml of active Akt (0.5 mg; Upstate Biotechnology) for 30 min at 30°C in 50 ml of buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM ATP, 150 mM KCl, 5 mM b-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM DTT). The reaction tubes contained either 1 mM amiloride or the same volume of DMSO. The reaction was stopped by adding 2× SDS sample buffer and heating at 100°C for 5 min. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analyzed by anti-phospho Akt antibody (Cell Signaling).

For in vitro PDK-1 kinase assays, LNCaP cells were transiently transfected with PDK-1 constructs (pcDNA3myc-PDK-1; 2 μg of DNA/dish) using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). One day after transfection, cells were lysed with 500 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Trition X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl,

10 mM NaF, 1 mM DTT, 1 mM sodium ortho-vanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma)). Cell lysates were immunoprecipitated by incubation with mouse anti-myc antibody (9E10; Roche, Indianapolis, IN) and protein G-Plus agarose (Gibco BRL) for 2 h at 4°C. Immune complexes were washed twice with buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 mM DTT, 1 mM Na_3VO_4, and 1 mM PMSF) at 4°C and then incubated with 2 µg of purified inactive Akt protein (Upstate Biotechnology) in a volume of 50 µl of a kinase buffer (100 µM ATP, 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM EDTA) for 30 min at 30°C. Thereafter, the sample was subjected to SDS–PAGE and the phosphorylation of Akt was analyzed by rabbit anti-phospho-Akt antibody (Cell Signaling).

RESULTS

TRAIL in Combination With Amiloride Induces Cytotoxicity

To investigate the effect of amiloride on TRAIL-induced cytotoxicity, human prostatic adenocarcinoma LNCaP cells were treated with TRAIL in the presence or absence of amiloride. Little or no cytotoxicity was observed with TRAIL (1–200 ng/ml) alone (Fig. 1A) or amiloride (0.1–1 mM) alone (Fig. 1B). In contrast, TRAIL in combination with amiloride significantly induced cytotoxicity (Fig. 1C). TdT-mediated dUTP Nick end labeling (TUNEL) assay showed that apoptotic death occurred during combined treatment with TRAIL and amiloride (Fig. 1D). Similar results were observed by DAPI staining (Fig. 1E). DAPI staining of cells treated TRAIL in combination with amiloride showed the presence of many cells with condensed nuclei, a morphological change that is associated with apoptosis.

Combined Treatment With TRAIL and Amiloride Induces Apoptosis

Additional studies were designed to examine whether the combination of amiloride and TRAIL treatment in LNCaP cells enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis. Previous studies show that PARP (116 kDa) is cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL in human prostate adenocarcinoma DU-145 cells [Lee et al., 2004]. Figure 2A,B shows that the cleavage of PARP was not
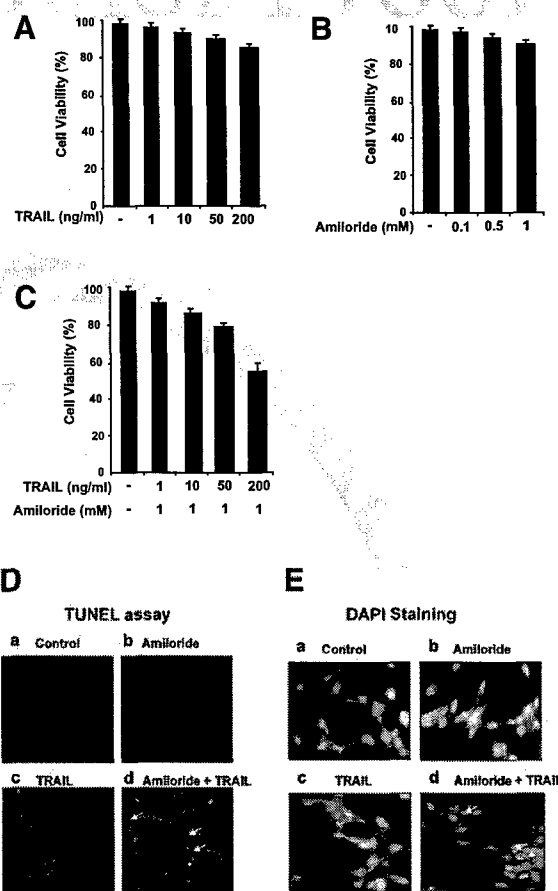
Fig. 1. Effect of amiloride on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells. **A:** Cells were treated for 4 h with various concentrations of TRAIL (1–200 ng/ml). **B:** Cells were treated with various concentrations of amiloride (0.1–1 mM). **C:** Cells were treated with various concentrations of TRAIL (1–200 ng/ml) in the presence of 1 mM amiloride. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **D:** Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by arrows. **a:** Untreated control; **(b)** amiloride; **(c)** TRAIL; **(d)** amiloride + TRAIL. **E:** Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, cells were stained with DAPI (1 µg/ml), and morphological features were analyzed with a fluorescence microscope.

observed by treatment with amiloride (0.1–1 mM) alone or TRAIL (1–1,000 ng/ml) alone in LNCaP cells. Interestingly, PARP was cleaved by combined treatment with TRAIL (50–1,000 ng/ml) and amiloride (0.5–1 mM). Similar results were observed with HMA, an analogue of amiloride (Fig. 2C). Figure 2D demonstrates that TRAIL in combination with amiloride, but

not TRAIL alone, activated caspases. Amiloride alone did not activate caspases (data not shown). However, Western blot analysis shows that procaspase-8 (54/55 kDa) was cleaved to the intermediates forms (41 and 43 kDa) and active form (18 kDa) by treatment with TRAIL in the presence of amiloride. The combined treatment of TRAIL and amiloride also resulted in caspase-9 activation (Fig. 2D). TRAIL in combination with amiloride induced proteolytic processing of procaspase-9 (48 kDa) into its

active form (37 kDa). The combined treatment with TRAIL and amiloride also induced caspase-3 activation. Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) in the presence of TRAIL and amiloride.

Effect of Combined Treatment With TRAIL and Amiloride on the Level of TRAIL Receptor Family and Anti-Apoptotic Proteins
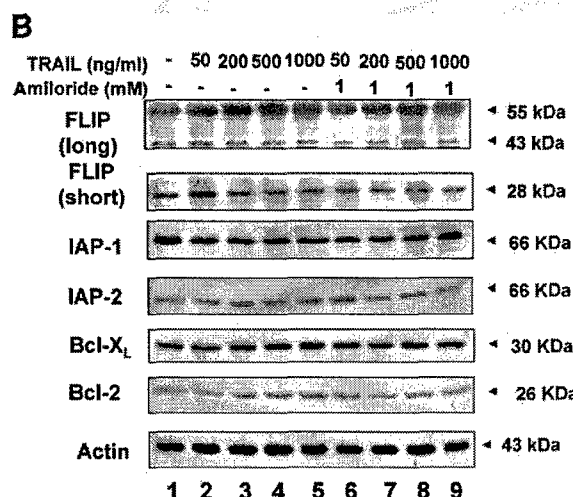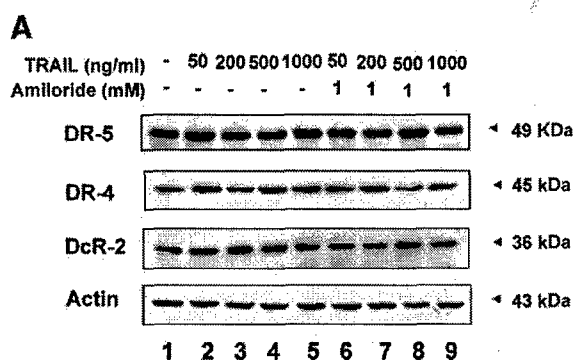
Previous studies demonstrate that increased DR5 levels induced by genotoxic agents [Sheikh et al., 1998; Chinnaiyan et al., 2000] or decreased FLIP expression induced by glucose deprivation [Nam et al., 2002] is responsible for increasing TRAIL cytotoxicity. Thus, we examined whether changes in the amounts of TRAIL receptors and anti-apoptotic proteins are associated with the promotion of apoptosis by TRAIL in combination with amiloride. LNCaP cells were treated with TRAIL (50–1,000 ng/ml) in the presence of 1 mM amiloride. Data from western blot analysis reveal that the combined treatment did not significantly alter the levels of DR4, DR5, DcR2, $FLIP_L$, $FLIP_S$, IAP-1, IAP-2, $Bcl-X_L$, and Bcl-2 (Fig. 3). Amiloride alone also did not change the levels of TRAIL receptors and anti-apoptotic proteins (data not shown).

Effect of Amiloride on Akt Phosphorylation

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis [Nesterov et al., 2001]. Since several researchers have also reported that overexpression of Na^+/H^+ exchanger promotes cell survival and Akt activity [Barriere et al., 2001; Wu et al.,
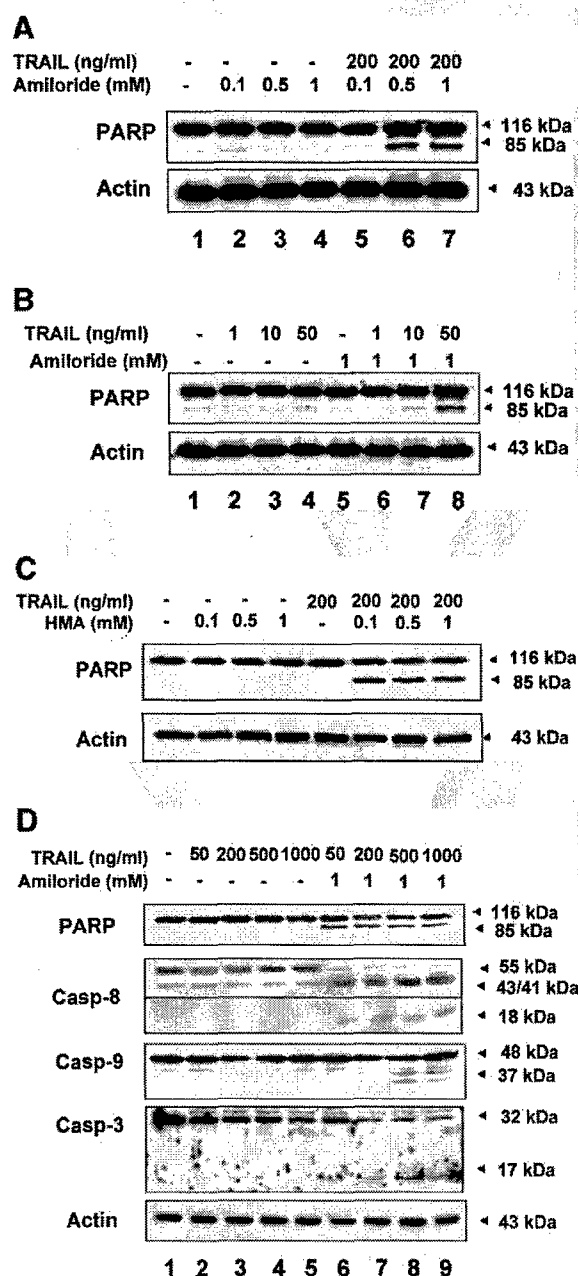
Fig. 2. Effect of amiloride or HMA on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP cells. Cells were treated for 4 h with various concentrations of amiloride (0.1–1 mM) in the presence or absence of 200 ng/ml TRAIL (A), various concentrations of TRAIL (1–50 ng/ml) in the presence or absence of 1 mM amiloride (B), various concentrations of HMA (0.1–1 mM) in the presence or absence of 200 ng/ml TRAIL (C), or various concentrations of TRAIL (50–1,000 ng/ml) in the presence or absence of 1 mM amiloride (D) and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (41, 43 kDa), and active form (18 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

A

TRAIL (ng/ml) - 50 200 500 1000 50 200 500 1000
Amiloride (mM) - - - - - 1 1 1 1

DR-5 ◄ 49 KDa

DR-4 ◄ 45 kDa

DcR-2 ◄ 36 kDa

Actin ◄ 43 kDa

1 2 3 4 5 6 7 8 9

B

TRAIL (ng/ml) - 50 200 500 1000 50 200 500 1000
Amiloride (mM) - - - - - 1 1 1 1

FLIP ◄ 55 kDa
(long) ◄ 43 kDa

FLIP ◄ 28 kDa
(short)

IAP-1 ◄ 66 KDa

IAP-2 ◄ 66 KDa

Bcl-X$_L$ ◄ 30 KDa

Bcl-2 ◄ 26 KDa

Actin ◄ 43 kDa

1 2 3 4 5 6 7 8 9

Fig. 3. Intracellular levels of TRAIL receptors (A) or anti-apoptotic proteins (B) during treatment with TRAIL in the presence or absence of amiloride. LNCaP cells were treated for 4 h with various concentrations of TRAIL (50–1,000 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 µg) were separated by SDS–PAGE and immunoblotted as described in "Materials and Methods." Actin is shown as an internal standard.

1 mM Amiloride

C 5 10 20 30 60 120 180 240 (min)

p-Akt 60 kDa

Akt 60 kDa

Actin 42 kDa

1 2 3 4 5 6 7 8 9

Fig. 4. Effect of amiloride on the level of phosphorylated Akt in LNCaP cells. Cells were treated with 1 mM amiloride for various times (5–240 min) and then harvested. Equal amounts of protein (20 µg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, or anti-Akt antibody. Actin is shown as an internal standard.
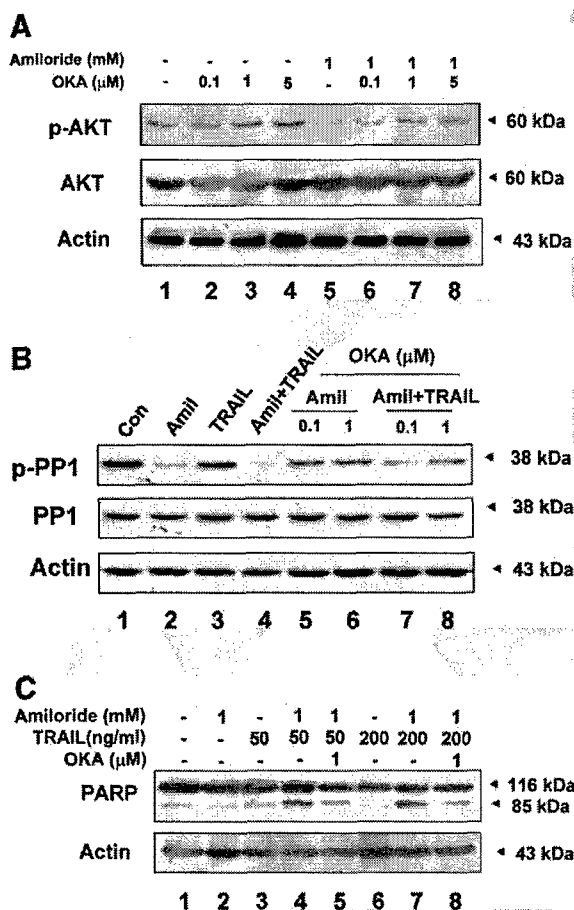
2004], we postulated that amiloride inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity. To examine whether amiloride inhibits Akt activity by dephosphorylating Akt, LNCaP cells were treated with 1 mM amiloride for various periods (5–240 min) and the level of phosphorylated Akt was measured. Figure 4 shows that Akt was rapidly dephosphorylated within 30 min of amiloride addition without changing the Akt protein level. TRAIL treatment did not alter amiloride-induced dephosphorylation of Akt (data not shown). Since amiloride is acting as a Na^+/H^+ exchanger inhibitor, promoting intracellular acidification may inhibit Akt activity. However, low extracellular pH does not dephosphorylate Akt (data not shown). To examine whether dephosphorylation of Akt during amiloride treatment is mediated through activation of phosphatase, we treated LNCaP cells with okadaic acid, a serine phosphatase inhibitor. Figure 5A shows that amiloride-induced Akt dephosphorylation (lane 5) was suppressed by pretreatment with 0.1–5 µM okadaic acid (lanes 6–8), but not with low concentrations (<0.1 µM) of okadaic acid (data not shown). Since okadaic acid inhibits both protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), (both serine/threonine phosphatases), at higher concentrations (IC_{50} = 150 nM), but it inhibits only PP2A at low concentrations (IC_{50} < 0.1 nM), PP1 rather than PP2A may play an important role in the regulation of Akt phosphorylation during treatment with amiloride. Recent studies have also demonstrated that dephosphorylation of Akt is regulated by PP1, but not by PP2A [Xu et al., 2003]. Figure 5B shows that reduced phosphorylation of PP1 in the presence of amiloride (activation of PP1; lane 2) was partially antagonized by pretreatment with okadaic acid (lanes 5 and 6). Figure 5B also shows that amiloride in combination with TRAIL led to greater dephosphorylation of PP1 than amiloride alone and that this was not easily blocked by okadaic acid. These results indicate that reduction of phosphorylation is more responsible than stimulation of dephosphorylation during treatment with amiloride. We further examined whether okadaic acid prevents the combined treatment with TRAIL and amiloride-induced PARP cleavage (apoptosis). Amiloride-promoted TRAIL-induced PARP cleavage (lanes 4 and 7 in Fig. 5C) was significantly inhibited by treatment with 1 µM okadaic acid (lanes 5 and 8 in Fig. 5C).

A

| Amiloride (mM) | - | - | - | - | 1 | 1 | 1 | 1 |
| OKA (µM) | - | 0.1 | 1 | 5 | - | 0.1 | 1 | 5 |

p-AKT — ◄ 60 kDa

AKT — ◄ 60 kDa

Actin — ◄ 43 kDa

1 2 3 4 5 6 7 8

B

OKA (µM)

Con Amil TRAIL Amil+TRAIL

| | | | Amil | | Amil+TRAIL | |
| | | | 0.1 | 1 | 0.1 | 1 |

p-PP1 — ◄ 38 kDa

PP1 — ◄ 38 kDa

Actin — ◄ 43 kDa

1 2 3 4 5 6 7 8

C

Amiloride (mM)	-	1	-	1	1	-	1	1
TRAIL(ng/ml)	-	-	50	50	50	200	200	200
OKA (µM)	-	-	-	-	1	-	-	1

PARP — ◄ 116 kDa
◄ 85 kDa

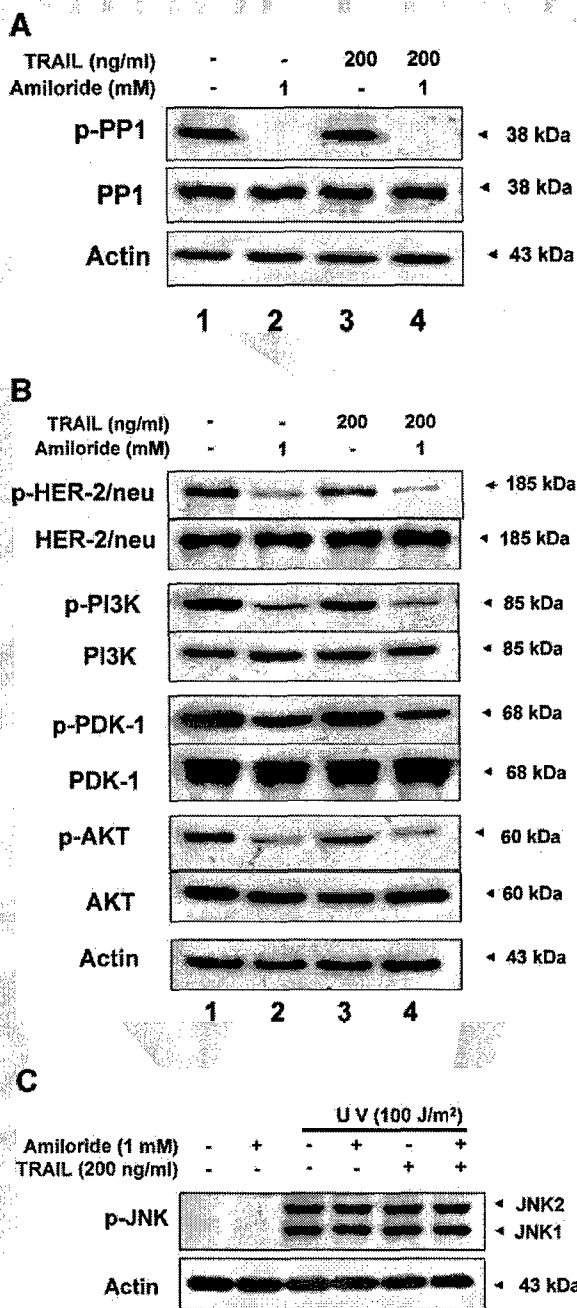Actin — ◄ 43 kDa

1 2 3 4 5 6 7 8

Fig. 5. Effect of okadaic acid on amiloride-induced PP1 dephosphorylation (A), Akt dephosphorylation (B), and TRAIL cytotoxicity (C). A: LNCap Cells were pretreated with okadaic acid (OKA: 0.1–5 µM) for 30 min and then treated or untreated with 1 mM amiloride. B: Cells were pretreated with okadaic acid (0.1–1 µM) for 30 min and treated with TRAIL in the presence or absence of 1 mM amiloride (Amil). Con, untreated control cells. C: Cells were pretreated with 1 µM okadaic acid for 30 min and treated with TRAIL (50–200 ng/ml) in the presence or absence of 1 mM amiloride. Equal amounts of protein (20 µg) were separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, anti-phospho-PP1, anti-PP1, or anti-PARP antibody. Actin is shown as an internal standard.

Effect of Amiloride on Kinases and Phosphatases Associated With the HER-2/neu-PI3K-Akt Pathway

Since previous studies have revealed that HER-2/neu activates the PI3K-Akt pathway, we further examined whether amiloride nonspecifically affects the HER-2/neu-PI3K-Akt pathway-associated kinases or phosphatases or specifically activates PP1 activity. As shown previously, amiloride induced dephosphorylation (activation) of PP1 (Fig. 6A). Interestingly,

A

| TRAIL (ng/ml) | - | - | 200 | 200 |
| Amiloride (mM) | - | 1 | - | 1 |

p-PP1 — ◄ 38 kDa

PP1 — ◄ 38 kDa

Actin — ◄ 43 kDa

1 2 3 4

B

| TRAIL (ng/ml) | - | - | 200 | 200 |
| Amiloride (mM) | - | 1 | - | 1 |

p-HER-2/neu — ◄ 185 kDa

HER-2/neu — ◄ 185 kDa

p-PI3K — ◄ 85 kDa

PI3K — ◄ 85 kDa

p-PDK-1 — ◄ 68 kDa

PDK-1 — ◄ 68 kDa

p-AKT — ◄ 60 kDa

AKT — ◄ 60 kDa

Actin — ◄ 43 kDa

1 2 3 4

C

UV (100 J/m²)

| Amiloride (1 mM) | - | + | - | + | - | + |
| TRAIL (200 ng/ml) | - | - | - | - | + | + |

p-JNK — ◄ JNK2
◄ JNK1

Actin — ◄ 43 kDa

Fig. 6. Effect of amiloride on phosphatase (A), kinases (B), or JNK (C) in the presence or absence of TRAIL. A, B: LNCaP cells were treated for 2 h with 1 mM amiloride in the presence or absence of 200 ng/ml TRAIL. C: LNCaP cells were pretreated with 1 mM amiloride for 30 min, irradiated with UV (100 J/m²), incubated for 1 h in the presence or absence of TRAIL (200 ng/ml)/amiloride (1 mM), and then harvested. Equal amounts of protein (20 µg) were separated by SDS–PAGE and immunoblotted as described in "Materials and Methods." Actin is shown as an internal standard.

amiloride also induced dephosphorylation (inactivation) of the HER-2/neu-PI3K-Akt pathway-associated kinases such as HER-2/neu, PI3K, and PDK-1 (Fig. 6B). These results suggest that amiloride nonspecifically induced dephosphorylation of the HER-2/neu-PI3K-Akt pathway-associated kinases as well as phosphatase. We further examined whether amiloride has an affect on phosphorylation of a substrate

A

that is not in the HER-2/neu-PI3K-Akt pathway. Figure 6C shows that UV exposure activated JNK (phosphorylation of JNK1 and JNK2) and its phosphorylation was not inhibited by treatment with amiloride.
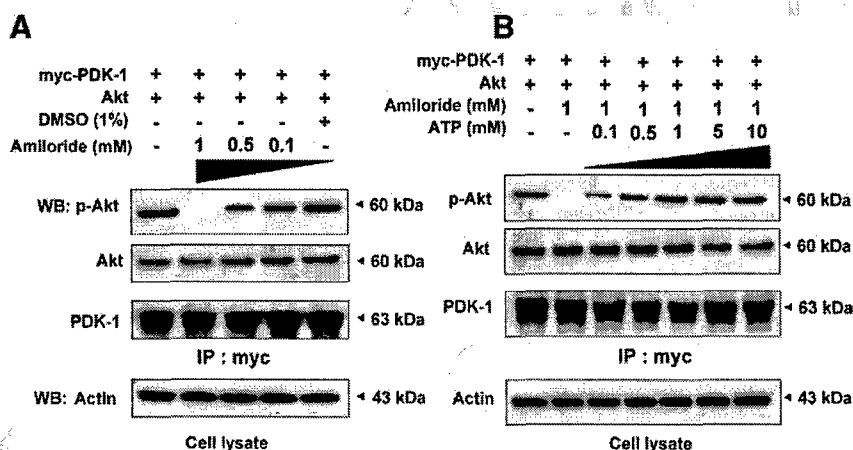
The effect of amiloride on Akt was compared with the effects of LY294002 and wortmannin, inhibitors of PI3K. Figure 7A shows that all these drugs induced dephosphorylation of Akt. However, unlike amiloride, LY294002 and wortmannin did not induce dephosphorylation of PI3K, PDK-1, and PTEN. We postulated that if amiloride-induced dephosphorylation of Akt is responsible for promoting TRAIL cytotoxicity, amiloride should enhance the effect of these kinase inhibitors. Indeed, amiloride in combination with LY294002 or wortmannin promoted TRAIL-induced cytotoxicity (Fig. 7B) and apoptosis (Fig. 7C). These results indicate that Akt inactivation (dephosphorylation) is responsible for the amiloride-induced enhancement of TRAIL cytotoxicity.

Mechanism of Amiloride-Induced Alterations of Phosphatase and Kinase Activities

Davis and Czech [1985] reported that amiloride acts as an ATP analogue which causes the formation of nonproductive enzyme–substrate complexes. We hypothesized that amiloride competes with ATP, thereby inhibiting protein phosphorylation. To test this hypothesis, we examined whether amiloride blocks the kinase-mediated phosphorylation process. Figure 8A shows that purified unphosphorylated Akt protein (Upstate Biotechnology; data not shown) was phosphorylated by active PDK-1 in vitro. Amiloride, but not DMSO, inhibited phosphorylation of Akt. Figure 8B shows that

B

C
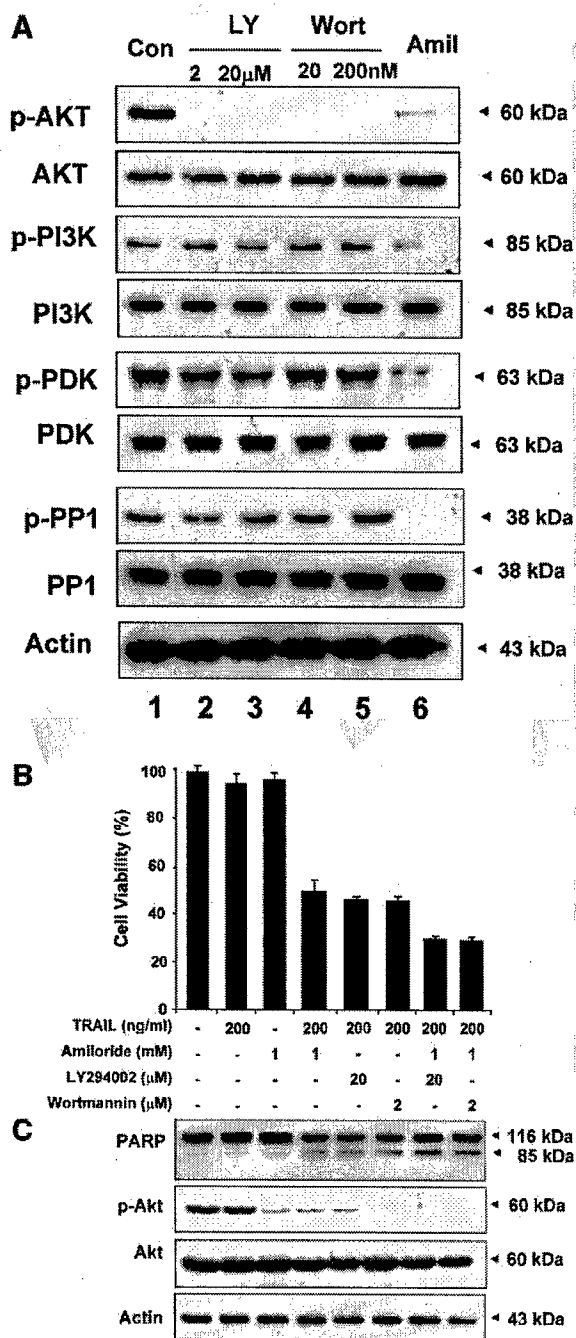
Fig. 7. Effect of LY294002 or wortmannin on kinases/phosphatases (A), TRAIL-induced cytotoxicity (B), and TRAIL-induced PARP cleavage (C) in LNCaP cells. A: Cells were treated for 1 h with LY294002 (LY; 2–20 μM), wortmannin (Wort; 20–200 nM), or 1 mM amiloride (Amil). Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in "Materials and Methods." Actin is shown as an internal standard. Con, untreated control cells. B, C: Cells were pretreated with 20 μM LY294002 or 200 nM wortmannin for 20 min and then treated with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride for 4 h. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-PARP, anti-phospho-Akt, or anti-Akt antibody. Actin is shown as an internal standard.

A

myc-PDK-1 + + + + +
Akt   + + + + +
DMSO (1%) - - - - +
Amiloride (mM) - 1 0.5 0.1 -

WB: p-Akt ◄ 60 kDa

Akt ◄ 60 kDa

PDK-1 ◄ 63 kDa

IP : myc

WB: Actin ◄ 43 kDa

Cell lysate

B

myc-PDK-1 + + + + + + +
Akt   + + + + + + +
Amiloride (mM) - 1 1 1 1 1 1
ATP (mM)   - - 0.1 0.5 1 5 10

p-Akt ◄ 60 kDa

Akt ◄ 60 kDa

PDK-1 ◄ 63 kDa

IP : myc

Actin ◄ 43 kDa

Cell lysate

Fig. 8. Effect of amiloride on PDK-1 kinase activity. LNCaP cells were transfected with pcDNA3myc-PDK-1. One day after transfection, myc-PDK-1 was immunoprecipitated with anti-myc antibody. For in vitro PDK-1 kinase assays, the immune complex was incubated with p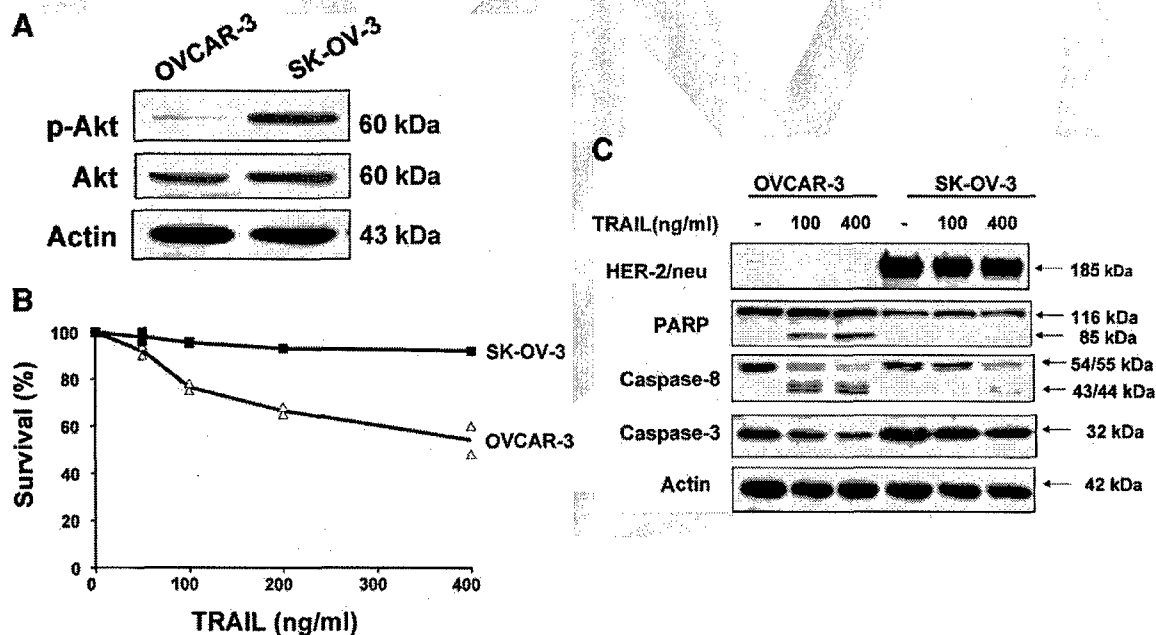urified inactive Akt protein in the presence or absence of various concentrations of amiloride (A) or various concentrations of ATP (B). The sample was separated by SDS–PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, or anti-PDK-1 antibody. Actin is shown as an internal standard.

amiloride blocks the kinase-mediated phosphorylation process by competing with ATP.

Ability of Amiloride-Induced TRAIL-Sensitivity Is not Specific to LNCaP Cells

To examine whether our findings can be generalized for more than one cell line, human

ovarian adenocarcinoma SK-OV-3 and OVCAR-3 cell lines were employed. Figure 9 shows that SK-OV-3 cells containing a relatively high level of Akt activity and HER-2/neu are resistant to TRAIL. In contrast, OVCAR-3 cells containing a relatively low level of Akt activity and HER-2/neu are sensitive to TRAIL. When SK-OV-3 or

A

OVCAR-3   SK-OV-3

p-Akt   60 kDa

Akt   60 kDa

Actin   43 kDa

B

C

OVCAR-3   SK-OV-3

TRAIL(ng/ml)   - 100 400 - 100 400

HER-2/neu ◄— 185 kDa

PARP ◄— 116 kDa
     ◄— 85 kDa

Caspase-8 ◄— 54/55 kDa
          ◄— 43/44 kDa

Caspase-3 ◄— 32 kDa

Actin ◄— 42 kDa

Fig. 9. Expression of HER-2/neu, basal Akt activity, and TRAIL sensitivity in ovarian adenocarcinoma SK-OV-3 and OVCAR-3 cells. A: Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-Akt antibody (Akt) or anti-phospho-S473 Akt antibody (p-Akt). Actin is shown as an internal standard. B: Cells were treated with TRAIL (0–400 ng/ml) for 4 h. Survival was analyzed by the trypan blue exclusion assay. Data from two separate experiments are compiled. C: Cells were treated with TRAIL (0–400 ng/ml) for 4 h. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HER-2/neu, anti-PARP, anti-caspase-8, anti-caspase-3, or anti-actin antibody.

OVCAR-3 cells were treated with 400 ng/ml TRAIL for 4 h, the survival was 92% or 60%, respectively. Similar results were observed when PARP cleavage and caspase (-8 and -3) activation were examined. TRAIL-induced activation of caspase-8 and caspase-3 as well as PARP cleavage were observed in OVCAR-3. Unlike OVCAR-3 cells, SK-OV-3 cells are resistant to TRAIL-induced PARP cleavage and caspase activation. However, the combined treatment of TRAIL and amilroide resulted in an increase in caspase (-8, -9, and -3) activation along with PARP cleavage in SK-OV-3 cells (Fig. 10). Amiloride dephosphorylated Akt regardless of the presence of TRAIL.
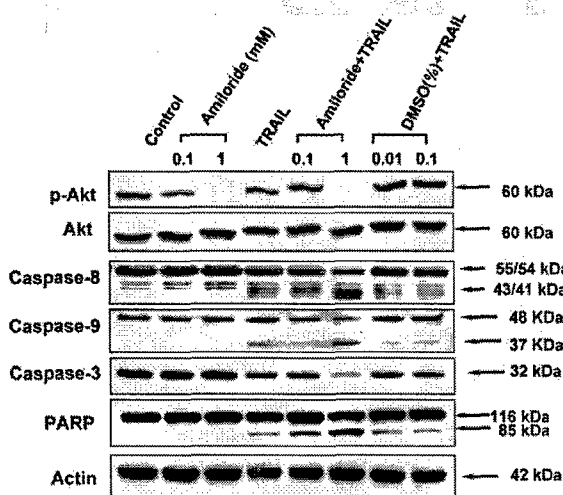
DISCUSSION

LNCaP and SK-OV-3 cells, which express high levels of HER-2/neu protein, are relatively insensitive to TRAIL and have a high level of Akt activation [Nesterov et al., 2001; Li et al., 2004; Fig. 9]. Previous studies demonstrated that down-regulation of the HER-2/neu protein and inhibition of Akt activity by treatment with trastuzumab (Herceptin) enhance TRAIL-mediated apoptosis [Cuello et al., 2001]. Similar results were observed by treatment with amiloride. Inactivation of HER-2/neu and Akt through treatment with amiloride results in

Fig. 10. Effect of amiloride on Akt activity and TRAIL-induced activation of caspases and cleavage of PARP in SK-OV-3 cells. Cells were treated with amiloride (0.1–1 mM) in the presence or absence of TRAIL (400 ng/ml) for 4 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-Akt, anti-phospho-S473 Akt, anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-PARP, or anti-actin antibody.

cytotoxicity by TRAIL (Figs. 1, 4, and 6). Our studies also reveal that amiloride causes a decrease in the phosphorylation state of Akt by inhibiting (dephosphorylation of) PI3K and PDK-1 kinases as well as activating (dephosphorylation of) PP1 (Fig. 6). The inhibition of kinases by amiloride can be overcome at high ATP concentrations which indicates that the inhibition of kinase activity is competitive with ATP (41; Fig. 8). Thus, amiloride may act as an ATP analogue and directly inhibit kinase activity and/or activate phosphatase activity by decreasing protein phosphorylation [Davis and Czech, 1985]. Indeed, our results from in vitro kinase assays show that amiloride directly inhibits PDK-1 activity (Fig. 8).

LNCaP cells which are PTEN-null due to mutations contain a high level of Bcl-2 [Huang t al., 2001]. This is probably because Bcl-2 expression is down-regulated by PTEN [Huang et al., 2001; Rosser et al., 2004]. Previous studies revealed that transient transfection of PTEN into the PTEN-null cells results in decreased levels of Bcl-2 [Huang et al., 2001] and sensitizes cells to apoptotic agents [Huang et al., 2001; Yuan and Whang, 2002]. In this study, we observed that combined treatment with TRAIL and amiloride does not alter the level of Bcl-2 (Fig. 3B). These results suggest that amiloride-promoted TRAIL cytotoxicity is not mediated through Bcl-2. Previous studies show that amiloride inhibits tyrosine kinase activity of growth factor receptors [Davis and Czech, 1985], Na^+/Ca^{2+} exchange [Smith et al., 1982], (Na^+-K^+)-ATPase [Soltoff and Mandel, 1983], and serine kinase activity [Ralph et al., 1982]. It has previously been suggested that a possible mechanism of biochemical action of amiloride is the inhibition of kinase activity [Ralph et al., 1982; Davis and Czech, 1985]. In addition to the inhibition of kinase activity, our data indicate that amiloride activates PP1 phosphatases (Fig. 6A).

Previous studies showed that Akt activity was also regulated by Ser/Thr phosphatases such as PP2A [Resjo et al., 2002] or PP1 [Xu et al., 2003]. However, pharmacokinetic studies reveal that PP1 rather than PP2A is responsible for the regulation of Akt phosphorylation [Xu et al., 2003]. It is well known that the activity of PP1 is regulated by phosphorylation of its catalytic subunit. Upon phosphorylation of Thr320, the COOH terminus of PP1 folds back to mask its catalytic center [Goldberg et al.,

1995]. Our data clearly demonstrate that amiloride dephosphorylates (activates) PP1 (Figs. 5B and 6A) as well as Akt (Figs. 4 and 5A). The amiloride-mediated dephosphorylation of PP1 and Akt is inhibited by okadaic acid (Fig. 5). Previous pharmacokinetics studies demonstrate that okadaic acid inhibits only PP2A at low concentrations ($IC_{50} < 0.1$ nM), but both PP1 and PP2A at higher concentrations ($IC_{50} = 150$ nM). Thus, we need to further investigate whether PP2A is also involved in the regulation of Akt phosphorylation.

Previous studies have shown that constitutively active Akt blocks TRAIL cytoxicity [Chen et al., 2001; Thakkar et al., 2001]. Down-regulation of Akt activity by PI3K inhibitors, wortmannin and LY294002, promotes TRAIL cytotoxicity [Thakkar et al., 2001; Fig. 7]. This is probably due to inhibition of Akt-mediated antiapototic effects. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspasase-9, and Forkhead transcription factors [Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998; Brunet et al., 1999; Hetman et al., 2000]. The proapoptotic function of these molecules is suppressed upon phosphorylation by Akt. Akt also induces the degradation of IκB by promoting IKKα activity and subsequently stimulating the nuclear translocation of NF-κB [Ozes et al., 1999]. Recently we [Nam et al., 2002] and Panka et al. [2001] reported that the PI(3)K-Akt-NF-κB pathway may regulate the expression of FLICE-inhibitory protein (FLIP), an anti-apoptotic molecule. We postulate that amiloride-mediated dephosphorylation of Akt leads to activation of proapoptotic molecules and results in enhancement of TRAIL-induced apoptotic death. Overall, our model may provide important insights into how amiloride promotes TRAIL-induced apoptotic death. We believe that this model provides a framework for future studies.

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